BACKGROUND OF THE INVENTION

Citations to some documents may be indicated as numbers in parentheses; those numbers refer to the bibliography under the heading "Related Art" at the end of this section. Those references, as well as others cited in this document are hereby incorporated by reference.

Live bacterial vaccine vectors have been used successfully to elicit effective immune responses in order to prevent infection. Such vectors have been used to induce protective immunity against infection from homologous and heterologous bacterial strains. Live attenuated bacterial vectors are also useful for food safety, for example to prevent or reduce infection of livestock animals such as poultry or cattle by bacterial strains that are pathogenic to humans, such as *Salmonella* or *E. coli*.

The ability of live attenuated pathogenic bacteria of the Enterobacteriaceae family to colonize the gut-associated lymphoid tissue (GALT; Peyer's patches) and the deep tissues following oral administration is beneficial in that it stimulates all arms of the immune response, including mucosal, humoral and cellular immunities (Curtiss/Doggett/Nayak/Srinivasan 1996; Galan and Sansonetti 1996; Medina/Guzman 2001). Colonization of the intestinal tract by gram negative bacteria is dependent in part upon the expression of a number of surface antigens, including LPS O-antigen side chains, a diversity of fimbrial adhesins, flagella and certain outer membrane proteins. Thus, rough mutants, i.e., those with little or no O-antigen on their LPS, that have mutational lesions precluding synthesis of LPS O-antigen or parts of the LPS core tend not to colonize the intestinal tract (Roantree, 1971; Nnalue, 1990) and are defective in attaching to and invading intestinal cells and surviving in cells on the other side of the intestinal wall barrier. (25, 26). This latter phenotype is due to the fact that LPS is needed for bacteria to display resistance to killing by macrophages (27, 28) and also for the display of serum resistance (29, 30), that is, the ability to multiply in blood. In accord with these observations, rough mutants defective in LPS synthesis and thus defective in infection are among the most frequently isolated using signature tagged mutagenesis (31) and genes for LPS biosynthesis are very often up-regulated during infection as revealed by use of in vivo expression technology (32). Rough mutants have generally not been very effective when used as live vaccines. (33, 34, Hill

abstract). Thus, it follows that an attenuated immunogenic live bacterial vaccine, to be safe and efficacious must not only display avirulence and not induce disease symptomology, but also must be able to reach, multiply and persist for a while in those lymphoid organs necessary to stimulate a protective immune response. Permanently rough strains cannot achieve the latter. The use of bacterial strains with mutations in the *galE* locus encoding UDP-galactose epimerase, an enzyme that interconverts UDP-glucose and UDP-galactose (UDP-gal) (35), has been considered as a way of overcoming the above limitation. UDP-gal is needed for the synthesis of both the LPS core and O-antigen in many bacterial strains. (36). When *Salmonella galE* mutants are provided low levels of galactose, they make normal LPS, but when deprived of galactose, they rapidly lose the ability to synthesize a complete LPS O-antigen and core. (37). One of the difficulties with *galE* mutants is that they are exceedingly sensitive to galactose (38, 39) and accumulate Gal-resistant mutants that are permanently rough and therefore not only avirulent, but also non immunogenic. Because of the LPS core defect, these *galE* mutants are somewhat hyper attenuated and do not induce high-level protective immunity. (40, 41). Another alternative to generate a reversibly rough phenotype is to make use of *pmi* mutants that have a mutation in the gene for phosphomannose isomerase (42), which interconverts mannose 6-phosphate and fructose 6-phosphate. Mannose 6-phosphate is then converted to GDP-mannose which is used for synthesis of O-antigen side chains (43). *pmi* mutants are not mannose sensitive and, as shown by Collins et al. (44), are attenuated and somewhat immunogenic. *pmi* mutants, when grown in media containing mannose, synthesize wild-type levels of LPS O-antigen side chains. In addition, *pmi* mutants do not lose the ability to synthesize LPS core.

Immune responses to iron-regulated outer membrane proteins (IROMPS) are known to be effective in preventing septicemic infection with enteropathogens. (Bolin 1987). Further, many bacterial serotypes and species in the Enterobacteriaceae family synthesize IROMPs and other proteins involved in iron uptake that share significant antigenic homology such that antibodies induced to proteins from one bacterial serotype or species are effective in binding to IROMPS and other iron uptake proteins from other serotypes and species. (Jun Lin 2001).

The *fur* gene encodes a repressor that represses all genes encoding IROMPS, in the presence of free iron. (Earhart 1996). When iron concentrations become low, as is the case in most animal host tissues beyond the intestinal wall barrier, the *fur* repression decreases and higher level expression of IROMPS and other *fur*-regulated genes needed to sequester iron is observed. *fur* mutants are attenuated when fed orally, giving a two to three log higher LD50 when administered either to mice (52) or day-of-hatch chicks. On the other hand, administering a *fur* mutant of S. typhimurium by the intraperitoneal route leads to only a slightly elevated LD50 compared to that of the wild-type parent. (53). In the intestinal tract iron is plentiful, both due to non absorption of dietary iron and the presence of iron from hemoglobin breakdown contributed into the intestinal tract as a component of bile. Green et al. 1968. It is also well known that iron, unless in a complex form, can promote the formation of damaging hydroxyl radicals, which may account, in part, for the toxicity of iron (51). Thus the high oral LD50 of *fur* mutants may be due to toxicity of free iron encountered in the intestinal tract. *fur* mutants are also acid sensitive (55) and are thus potentially sensitive to the gastric acidity barrier and to killing in acidified phagosomes in macrophages (56, 57). In summary, while *fur* mutant bacterial strains would display higher levels of IROMPs that likely would induce protective immunity, their avirulence properties when administered orally make them poor immunogens. So, while mutants unable to produce Fur are attenuated when delivered orally, because of substantial iron induced death they do not induce a significant immune response.

Members of the Enterobacteriaceae family cause a wide variety of human and animal diseases, including gram-negative sepsis, food poisoning, and typhoid fever. In addition, many farm animals are colonized with diverse enteric bacteria such as many serotypes of Salmonella without causing disease. Such bacteria are capable of transmission through the food chain to cause diseases in humans. Developing vaccines to prevent all the types of enteric diseases caused by bacterial enteric pathogens of diverse genera, species and serotypes and to prevent colonization by these diverse bacterial types in farm animals to enhance food safety would be prohibitively expensive. The incidence of these diseases and the prevalence of colonization of farm animals highlights the need for vaccines that would cross-protect against the

numerous species and serotypes of enteric bacteria. Thus, it would be useful to develop attenuated bacterial vaccine strains that are capable of inducing cross-protective immunity.

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SUMMARY OF THE INVENTION

The inventors have discovered that by combining, in a live attenuated derivative of an Enterobacteriaceae, a genetic construction that allows regulated expression of a regulatory protein such that antigenic proteins which are conserved among Enterobacteriaceae are expressed *in vivo*, and a means for regulatable synthesis of LPS O-antigens such that said O-antigens cease to be expressed *in vivo*, said live attenuated derivative has enhanced ability to induce cross-protective immunity against a diversity of gram negative pathogens. As used herein, the term "pathogen" refers to organisms that cause disease symptoms in an animal. A pathogen need not necessarily cause disease symptoms in the animal to which the live attenuated derivative is administered. For example, many *Salmonella* serotypes are not pathogens for chickens and swine, but persist commensally, and then become pathogens in humans when transferred through the food chain. Thus, the term pathogen as used herein would apply to such *Salmonella* serotypes.

The inventors have shown that the above described live attenuated derivatives are effective in colonizing in the intestinal tract of an individual and invading into lymphoid tissue such that a high-level immune response is induced which protects the individual from infection from a diversity of species or serotypes of bacterial pathogens. A further advantage of such a

live attenuated derivative is that even when administered to an individual at exceedingly high doses, the risk of death is low.

In one embodiment of the invention, the regulatory protein is a ferric uptake regulator protein (Fur), which is encoded by the *fur* gene. The inventors have shown that by replacing the *fur* promoter with a regulatable promoter, the bacterial strain can be attenuated while still maintaining its immunogenicity. In a preferred embodiment of the invention, such regulated expression can be achieved by replacing the promoter for the *fur* gene with a metabolically controlled promoter such as that of the arabinose operon, the *araC*P_{BAD} activator-repressor-promoter system. In other embodiments, the regulatory protein may be, for example, the protein encoded by the *rpoS, phoPQ, dam, ompR, cya* or *crp* gene.

Synthesis of LPS O-antigen can be regulated by any means known in the art. For example, synthesis of O-antigen may be regulated by mutation of or regulation of any of the genes in the *rfb* gene cluster, or by mutation or regulation of RfaH or the JUMPstart sequence located upstream of the O-antigen gene cluster, or by mutation of or regulation of any of the other genes involved in regulation of any of the genes of the O-antigen gene cluster. (Iredell 1998; Wang 1998; Schnaitman 1993; Klena 1998; Kelly 1996). In one embodiment of the invention, synthesis of LPS O-antigen is regulated by means of a mutation in a *pmi* gene, which encodes phospho-mannose isomerase. Live attenuated derivatives harboring such a *pmi* mutation cannot synthesize LPS O-antigen side chains unless grown in the presence of free mannose. Thus, such mutants are unable to synthesize O-antigen side chains *in vivo*, as mannose in a free non-phosphorylated form is not prevalent in animal tissues. The presence of the *pmi* mutation leads to a gradual elimination of LPS O-antigen side chains *in vivo*, which then better exposes the LPS core and the IROMPs and other proteins involved in iron uptake, along with other surface proteins, which are conserved among genera and species within the Enterobacteriaceae family. Thus, the live attenuated derivative comprising the combination of the above described elements, when administered to an animal has enhanced ability to induce immune responses to IROMPs and other Fur regulated proteins and to the LPS core antigen to

confer cross-protection against infection by diverse genera species and serotypes of Enterobacteriaceae.

Some embodiments of the invention may further comprise a means for decreasing the expression of antigenic proteins and carbohydrates that show a great degree of diversity among the Enterobacteriaceae. These embodiments have the advantage of directing the immune response of the host animal to the conserved antigens, such that the cross-protective immunity is enhanced. Examples of such non-conserved antigenic proteins and carbohydrates include the flagella, LPS O-antigens, and fimbriae. In one embodiment, the *fliC* or *fljB* genes, which encode flagella are mutated. In another embodiment, both the *fliC* and *fljB* genes are mutated. In other embodiments the deletion mutations in the *fliC* and *fliB* genes only delete regions encoding antigenic variable domains and retain constant flagellar domains that induce T-cell immunity and recruit an inate immune response by interaction of the flagellar constant domains with the TLR5 receptor.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A and 1B illustrate the construction of a suicide vector for transfer of ΔPfur223::TT*araC*P_{BAD}*fur* deletion-insertion mutation.

FIG. 2 shows the ΔPfur223::TT*araC*P_{BAD}*fur* deletion-insertion chromosomal construction.

FIG. 3 illustrates the construction of a suicide vector for *pmi* deletion.

FIG. 4 shows the chromosomal deletion for Δ*pmi*-2426.

FIG. 5 demonstrates the reduction of LPS O-side chains in χ8650 as a function of time (hours) or numbers of generations of growth.

FIG. 6 demonstrates the outer membrane protein expression profile of ΔPfur223::TT *araC*P_{BAD}*fur* mutants grown in nutrient broth +/- arabinose.

FIG. 7 is a graphic illustration of colonization of Peyer's patches and spleens in 8-week-old female BALB/c mice as a function of time after oral inoculation with χ8634 ΔPfur::TT*araC*P_{BAD}*fur*.

FIG. 8 is a graphic illustration of colonization of Peyer's patches and spleens in 8-week-old female BALB/c mice as a function of time after oral inoculation with χ8650 Δ*pmi-2426*.

FIG. 9 is a graphic illustration of colonization of Peyer's patches and spleens in 8-week-old female BALB/c mice as a function of time after oral inoculation with χ8754 Δ*pmi-2426* ΔPfur223::*araC*P_{BAD}*fur*.

FIG. 10 illustrates the ability of χ8754, grown either in the presence or absence of mannose, to colonize the Peyer's patches and spleen of 8-week-old female BALB/c mice at designated intervals after oral inoculation.

FIG. 11 is a graphic illustration of the ability of serum antibodies collected from mice 30 days after oral inoculation with either χ8650 or χ8634 to react with the OMPs present in various Salmonella and E. coli strains grown in media containing excess iron such that the synthesis of IROMPs is minimal.

FIG. 12 is a graphic illustration of the ability of serum antibodies collected from mice 30 days after oral inoculation with either χ8650 or χ8634 to react with the IROMPS present in various Salmonella and E. coli strains grown in media substantially free of iron such that constitutive expression of fur-regulated proteins occurs.

FIG. 13 is a graphic illustration of colonization of day-of-hatch chicks as a function of time after oral inoculation with χ8754 Δ*pmi-2426* ΔPfur223::*araC*P_{BAD}*fur*.

FIG. 14 illustrates construction of the suicide vector for transfer of Δ*fliC825* deletion mutation.

FIG. **15** illustrates construction of a suicide vector for transfer of Δ*fljB217* deletion mutation.

FIG. **16** shows the Δ*fliC825* (A) and Δ*fljB217* (B) chromosomal deletion mutations.

FIG. **17** illustrates construction of a suicide vector for transfer of Δ*fliC*-Var mutation.

FIG. **18** illustrates construction of a suicide vector for transfer of Δ*fliC* 2426 mutation.

FIG. **19** shows *S. typhimurium* UK-1 chromosomal map for Δ*fliC*-Var and Δ*fliC* 2426 deletion mutations.

FIG. **20** shows the DNA nucleotide sequence of improved *araC** P_{BAD} region in pYA3624.

FIG. **21** shows the DNA and amino acid sequences of P_{fur} and *fur* gene of *S. paratyphi* A.

FIG. **22** illustrates construction of the suicide vector to introduce new ΔPfur-33::TT *araC* P_{BAD} *fur* deletion-insertion mutation.

FIG. **23** shows a chromosomal map of ΔPfur-33::TT *araC* P_{BAD} *fur* deletion-insertion mutation.

FIG. **24** shows the DNA sequence of the ΔPfur-33::TT *araC** P_{BAD} *fur*.

FIG. **25** shows the DNA and amino acid sequences of P_{rpoS}, *rpoS* and flanking region of *S. typhimurium* and *S. typhi*.

FIG. **26** illustrates construction of suicide vector for introducing ΔPrpoS-183::TT *araC* P_{BAD} *rpoS* deletion-insertion mutation.

FIG. **27** shows a chromosomal map of ΔPrpoS-183::TT *araC* P_{BAD} *rpoS* deletion-insertion mutation.

FIG. **28** shows the DNA and amino acid sequences of the *S. typhimurium* P_{phoPQ} and *phoPQ* and the flanking region.

FIG. **29** illustrates construction of the suicide vector for introducing ΔP_{phoPQ}-107::TT *araC* P_{BAD} *phoPQ* deletion-insertion mutation.

FIG. **30** shows a chromosomal map of ΔP_{phoPQ}-107::TT *araC* P_{BAD} *phoPQ* deletion-insertion mutation.

FIG. **31**shows suicide vectors for introducing the Δ*araBAD23* and Δ*araE25* deletion mutations.

FIG. **32** illustrates construction of the suicide vector for introducing the Δ(*gmd-fcl*)-26 deletion mutation.

FIG. **33** shows a chromosomal map of the Δ(*gmd-fcl*)-26 deletion mutation.

FIG. **34** shows diagrams of the suicide vectors shown in Table 2.

FIG. **35** illustrates various deletion mutations after insertion into *Salmonella* chromosome.

FIG. **36** shows the DNA and amino acid sequences of *sopB* and the flanking region of the *S. typhimurium* chromosome.

FIG. **37** illustrates construction of the suicide vector for introducing the Δ*sopB* deletion mutation into the *Salmonella* chromosome.

FIG. **38** shows a chromosomal map of Δ*sopB* deletion mutation.

FIG. 39 shows diagrams of the suicide vectors for introducing Δ*asdA16* into *S. typhimurium* and Δ*asdA25* into *S. paratyphi* A and S. typhi strains.

FIG. 40 shows chromosomal maps of Δ*asdA16* and Δ*asdA25* deletion mutations.

FIG. 41 shows maps of Asd^+ vectors with pSC101, p15A, pBR and pUC origins of replication to regulate plasmid copy numbers.

FIG. 42 shows the nucleotide sequence of P_{trc} and the multiple cloning sites (MCS) of Asd^+ vectors in FIG. 41.

FIG. 43 shows a diagram of the suicide vector for introducing Δ*ilvG3*::TT *araC* P_{BAD} *lacI* TT deletion-insertion mutation and map of Δ*ilvG3*::TT *araC* P_{BAD} *lacI* TT mutation in the *Salmonella* chromosome.

FIG. 44 shows the nucleotide and amino acid sequences of *S. typhimurium fimH* and FimH protein.

FIG. 45 illustrates construction of *fimH* Asd^+ vectors.

DESCRIPTION OF THE INVENTION

The invention is directed to live attenuated strains of Enterobacteriaceae that are capable of inducing cross-protective immunity to a diversity of Enterobacteriaceae species and serotypes. This objective has been achieved by the means and methods described herein.

The Enterobacteria family comprises species from the following genera, any of which are considered to be useful in practicing the claimed invention: *Alterococcus, Aquamonas, Aranicola, Arsenophonus, Brenneria, Budvicia, Buttiauxella, Candidatus Phlomobacter, Cedeceae, Citrobacter, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Hafnia,*

Klebsiella, Kluyvera, Leclercia, Leminorella, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Photorhabdus, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Salmonella, Samsonia, Serratia, Shigella, Sodalis, Tatumella, Trabulsiella, Wigglesworthia, Xenorhbdus, Yersinia, Yokenella. Due to their clinical significance, *Escherichia coli, Shigella, Edwardsiella, Salmonella, Citrobacter, Klebsiella, Enterobacter, Serratia, Proteus, Morganella, Providencia* and *Yersinia* are considered to be particularly useful. Some embodiments of the instant invention comprise species of the *Salmonella* genera, as this genera has been widely and extensively studied and characterized.

The LPS of *Enterobacteriaceae* comprises three distinct domains: 1) the O-specific polysaccharide (O-antigen); 2) the core oligosaccharide (consisting of the inner and outer core oligosaccharides); and 3) the lipid A. LPS is both a major virulence factor and a target for protective immune responses. The core region of LPS is highly conserved, in contrast to the O-antigen which is the basis for distinguishing the various serotypes of many Enterobacteriaceae species. In *Salmonella*, for example, over 2,000 serotypes have been identified on the basis of the diversity of their O-antigen type and their flagella type. In contrast, those serotypes of *Salmonella* share only two closely related LPS core types.

The ability of Enterobacteriaceae to colonize the intestinal tract of animals is dependent upon, among other factors, the expression of a number of surface antigens, including LPS O-antigen side chains, a diversity of fimbrial adhesins, flagella and other outer membrane proteins. LPS O-antigens are antigenically diverse as between strains of Enterobacteriaceae, and are a major factor in the variable immune response of host organisms to different strains of bacteria. It is known in the art that bacterial strains defective in the ability to synthesize LPS O-antigen substantially lack the ability to colonize the intestinal tract and to attach to and invade intestinal cells and survive in cells on the other side of the intestinal wall (i.e., internal tissues).

Thus, the bacterial strains of the invention comprise a means for regulatable synthesis of LPS O-antigens, such that O-antigens are synthesized when the strain is grown in vitro, and O-antigens cease to be synthesized in vivo, i.e., when the bacterial strains are

administered to an animal. LPS O-antigen synthesis is dependent on a host of genes, including the genes of the *rfb* gene cluster. Regulation of synthesis of LPS O-antigens can be achieved by any suitable means. In some embodiments of the invention, regulation is achieved by mutations to or regulation of genes involved in synthesis of the O-antigens.

In some embodiments, the *pmi* gene is mutated such that the gene product is not expressed. The *pmi* gene encodes phosphomannose isomerase, a sugar transferase which inter converts mannose 6-phosphate and fructose 6-phosphate. In the process of O-antigen synthesis, mannose 6-phosphate is then converted to GDP-mannose which is then used for synthesis of O-antigen side chains. Thus, bacterial strains with a mutation which renders the *pmi* gene inoperable fail to produce O-antigen side chains. However, when such mutants are grown on media containing mannose, they are able to produce wild-type levels of O-antigen side chains. This is advantageous because of the important role that the LPS, including the O-antigen side chains, plays in the colonization of the gut and deep tissues of the animal. When the strain is administered to the animal, where free non-phosphorylated mannose is no longer available, the strain ceases to synthesize O-antigen side chain and over the course of several generations the strain no longer has significant levels of O-antigen associated with the cell wall, thus exposing the LPS core to enhance the immune response to this highly conserved antigen. Therefore, another advantage of the *pmi* gene mutation is that the mutation does not affect the ability of the strain to synthesize LPS core. Thus, the mutant strain can be grown on media containing mannose to maintain wild-type expression of O-antigen and then when administered to an animal, will continue to express wild-type levels of LPS core while at the same time expression of the O-antigen side chains will be significantly diminished, resulting in enhanced immune response of the animal to the LPS core and diminished immune response to the O-antigen side chain.

Other means of regulating the synthesis of O-antigen side chains are expected to achieve the same advantages as described above with respect to the *pmi* mutation. Those of ordinary skill in the art will be able to devise other means of regulated synthesis of O-antigen side chains that meet the criteria of the invention based on the knowledge in the art of the process

by which O-antigen is synthesized in Enterobacteriaceae. It is contemplated that those means are within the scope of the present invention. For example, the promoter for any of the *rfb* genes, which are needed for the synthesis of the LPS O-antigen, can be replaced with the *araC*P_{BAD} activator-repressor-promoter system so that expression of the particular *rfb* gene is dependant on the presence of arabinose supplied in media during growth of the vaccine.

The bacterial strains of the invention also comprise a genetic construction that allows regulated expression of a regulatory protein, such that antigenic proteins or carbohydrates which are conserved among the Enterobacteriaceae are expressed *in vivo*. Among the proteins or carbohydrates expressed in the cell membrane and wall of Enterobacteriaceae, some have been shown to be conserved to varying degrees among the various genera and species. For example, the LPS core and iron regulated outer membrane proteins (IROMPs) have been shown to be antigenically conserved among the Enterobacteriaceae.

IROMPS are encoded by a number of genes, the expression of which is controlled by a repressor protein (Fur) encoded by the *fur* gene. In the presence of iron, such as in the intestinal lumen, Fur represses the expression of IROMPs. In the absence of iron, such as for example in most animal host tissues beyond the intestinal wall barrier (internal tissues), Fur repression ceases, and thus IROMPs and other Fur-regulated genes are highly expressed. This level of IROMP expression in vivo can be reduced by the presence of glucose and/or H_2O_2 by the activation of the fur gene promoter by the Crp and OxyR positive regulators, respectively, to cause transcription of the fur gene. This sythesis of Fur causes a reduced level of IROMP sythesis even in the absence of iron. While *fur* mutants have been shown to be attenuated when administered orally to animals, such *fur* mutants may be susceptible to iron toxicity in the intestinal lumen due to non absorption of dietary iron and the presence of iron from hemoglobin breakdown contributed into the intestinal tract as a component of bile. In addition, unless in a complex form, iron can promote the formation of damaging hydroxyl radicals, which may account in part for the toxicity of iron. Further, since *fur* has been shown to play a role in the acid tolerance of Enterobacteriaceae, *fur* mutants are potentially sensitive to the gastric acidity barrier and to killing in acidified phagosomes in macrophages. All of these factors contribute to

the fact that while *fur* mutants would display high levels of IROMPs that induce cross protective immunity, the avirulence properties of such mutants make them poor immunogens.

Thus, some embodiments of the bacterial strains of the present invention comprise a genetic construction which allows for regulated expression of the *fur* gene, such that *fur* is expressed when the strain is grown *in vitro*, and in the intestinal lumen, but is not expressed when the bacterial strain is in the host tissue beyond the intestinal wall barrier. Thus, the bacterial strain exhibits wild-type repressed levels of IROMP expression during growth *in vitro* and during the initial stage of infection, i.e. when in the intestinal lumen. Then after colonization of the lymphoid organs beyond the intestinal wall barrier, the strain exhibits constitutive high-level expression of IROMPs and other Fur-regulated proteins independent of the presence of absence of iron, glucose or H_2O_2.

The regulated expression of the gene encoding a regulatory protein, structural protein or biosynthetic enzyme protein (as shown in the Examples) may be achieved by any means available in the art. For example, it is common practice to delete the wild type promoter associated with a particular gene and replace it with a promoter from the same or a different organism that is regulatable. In one embodiment of the present invention, the genetic construction is one in which expression of the *fur* gene is dependent upon the presence of arabinose. Arabinose can be supplied in culture media, and is also present in the intestinal tract of animals, as a component of plants which constitute a common part of animal diets. However, arabinose is not present in animal tissues beyond the intestinal wall barrier. This is achieved by replacing the *fur* promoter with the *araC*P_{BAD} activator-repressor-promoter system. The *araC*P_{BAD} activator-repressor-promoter is dependent on the presence of arabinose, which binds to the *araC* gene product to activate transcription from the P_{BAD} promoter. So, when the *araC*P_{BAD} activator-repressor-promoter is operatively linked to the *fur* gene, in place of the *fur* promoter, expression of the *fur* gene is then dependent on the presence or absence of arabinose. For example, when the bacterial strain harboring such a genetic construction is grown in media supplemented with arabinose, or alternatively when the strain is in the lumen of the intestinal tract of an animal where arabinose is present, the *fur* gene is expressed and the expression

IROMPs and other *fur* regulated proteins is repressed. On the other hand, when such a bacterial strain invades the tissue on the other side of the intestinal wall barrier, where arabinose is absent, the *fur* gene is no longer expressed leading to high level of expression of all of the *fur* regulated proteins including IROMPs. The elimination of the *fur* gene promoter also eliminates any influence of either glucose or products of oxidative metabolism in reducing the level of synthesis of *fur* regulated proteins including IROMPs.

Some embodiments of the bacterial strains of the invention comprise mutations in genes that encode other antigenic proteins expressed on the surface of *Enterobacteriaceae*, but which proteins are not antigenically conserved among the genera and species of the *Enterobacteriaceae* family. Such mutations cause diminished expression of those proteins, such that the host immune response is focused on the conserved antigenic proteins and carbohydrate antigens, further enhancing cross-protective immunity. It is important that such mutations be selected such that the diminished expression of the particular gene product does not significantly inhibit the bacterial strain's ability to colonize the intestinal tract and invade and persist in the tissue beyond the intestinal wall barrier. Examples of other surface proteins that are not antigenically conserved among the *Enterobacteriaceae* include flagella, pili, and fimbriae among others. Some embodiments of the bacterial strains of the invention comprise genetic constructions that diminish the expression of flagella. In particular embodiments, the bacterial strains comprise mutations in the *fliC* or *fljB* genes, or both the *fliC* and *fljB* genes. Such mutations do not alter the ability of the bacterial strains to colonize the mucosal tissue of the animal or invade and persist in the tissue beyond the lumen of the intestine. It is expected, since the flagella are antigenically diverse among the Enterobacteriaceae, that such mutations will enhance the cross-protective immunity elicited by such strains upon administration to animals. This can be achieved by complete deletion of the *fliC* and *fljB* genes or by deleting only regions of the genes encoding antigenic variable domains. This enables retention of constant flagellar comains that induce T-cell immunity and recruit an innate immune response by interaction of the flagellar constant domains with the TLR5 receptor. The skilled artisan will appreciate that diminished expression of other surface proteins that are antigenically diverse will confer similar

characteristics as described with respect to the *fliC* and *fljB* mutations, thus achieving the same advantages as those mutations.

In a particular embodiment, the bacterial strains of the invention comprise a mutation in the *pmi* gene which renders that gene inoperable. A particularly preferred embodiment comprises the Δ*pmi-2426* mutation, which is described below in the Examples. The strain further comprises a genetic construction wherein the native *fur* gene promoter has been replaced by the *araC*P_{BAD} activator-repressor-promoter system. A particularly preferred embodiment comprises the ΔPfur223::TT *araC*P_{BAD}*fur* construction. A particularly preferred bacterial strain, which comprises the above mentioned genetic constructs is χ8754, the construction of which is described in detail in the Examples. The χ8754 strain exhibits wild-type levels of LPS O-antigen and wild-type repressed levels of IROMPs both during growth of the strain and during initial stages of infection of visceral organs whether administered orally or by course spray to young chickens. Then after colonization of visceral lymphoid organs, LPS O-antigen synthesis ceases and overexpression of IROMPs commences. Thus, this strain is attenuated, efficiently colonizes lymphoid tissues following oral administration to animals and induces high-level protective immunity to subsequent challenge with a plurality of wild-type *Enterobacteriaceae*.

In an alternative of the embodiment described immediately above, instead of mutating the *pmi* gene, the *pmi* promoter is replaced with the *araC*P_{BAD} activator-promoter. Thus, only after several generations of growth *in vivo* would LPS O-antigen cease.

Other embodiments, as shown in Example 19, comprise construction of candidate vaccine strains with mutational alterations that prevent display of motility to access food sources, ability to produce exopolysaccharide capsules that enhance survival, ability to make components of the extracellular matrix that enhance Biofilm formation and survival, reduce survival to starvation stresses and uncouple the necessity of protein synthesis to display any trait to prevent sustained survival of the vaccine strain in vivo or following excretion into the environment.

Other embodiments comprise the design and construction of vaccine strains of *S. typhimurium*, *S. paratyphi A* and *S. typhi* to be used to immunize humans and to include

mutations in such vaccines as described in Examples 20 and 21 to prevent vaccine induction of gastroenteritis in human vaccinees.

Other embodiments comprise means to use constructed vaccine strains to serve as antigen delivery vectors as described in Example 22, and to exhibit regulated delayed expression in vivo of protective antigens that are immunologically cross reactive and very similar on many enteric bacteria as described in Example 23, so as to enhance induction by the vaccine strain of cross-protective immunity to many enteric bacteria of differing serotypes and species.

The invention further comprises methods for inducing an immune response comprising administering any of the above described bacterial strains to an animal. Such bacterial strains may be administered by any means known in the art. Preferred methods of administration include, for example, oral administration, gastric intubation, or in the form of aerosols, for example by the whole-body spray method described in PCT publication WO 00/04920. Other methods of administration are also possible, for example by injection. Dosages required for induction of cross-protective immunity will vary, although routine experimentation will allow the skilled artisan to make such determinations. Pharmaceutical carriers, in which the bacterial strains are suspended are also known in the art.

Administration of the bacterial strains of the invention can be a single dose, or as is not uncommon, in a series of two or more doses. Such subsequent administrations of the bacterial strain are commonly referred to as boosters, and in many instances such boosters result in prolonged protection of the host animal.

The above disclosure describes several embodiments of the invention, and the examples below further illustrate the invention. The skilled artisan will recognize that other embodiments that provide the same advantages may also be employed in the practice of this invention. The scope of this invention is intended to be defined by the claims, and the description and examples are intended to be non-limiting.

EXAMPLES

Table 1 lists the bacterial strains referred to throughout the Description and Examples, and Table 2 lists the plasmids used in the following Examples.

Table 1. Bacterial Stains

Strain #	Strain	Phenotype/Genotype or	*Reference/ Source*
A. *Escherichia coli*			
DH5α	*E. coli* K-12	Δ(*lacZYA-arg F*)*U169* (ϕ80 *lacZ* ΔM15) *glnV44 recA1 endA1 gyrA96 thi-1 relA1 hsdR17*	1
MGN-617	*E. coli* K-12	SM10 λ*pir* Δ*asdA4* Δ*zhf-2*::Tn*10*	2
χ289	*E. coli* K-12	F- prototroph	3
χ6206	*E. coli* 026:H11	EPEC	S. Ashkenazi
χ6212	*E. coli* K-12	Δ*asdA4* Δ*zhf-2*::Tn*10* derivative	DH5α
χ7122	Avian *E. coli*	O78:K80:H9	4
χ7235	Avian *E. coli* TK3	O1:K1:H7	5
χ7302	Avian *E. coli* MT512	O2:K1:H+	6
B. *Salmonella enterica*			
χ3201	*S. agona* NR1	wild-type group B (1,4,12)	7
χ3202	*S. albany* NR2	wild-type group C_3 (8,20)	7
χ3203	*S. anatum* NR3	wild-type group E_1 (3,10)	7
χ3206	*S. bredeney* NR8	wild-type group B (1,4,12,27)	7
χ3210	*S. hadar* NR14	wild-type group C_2 (6,8)	7
χ3212	*S. heidelberg* NR99	wild-type group B (1,4,5,12)	7
χ3213	*S. infantis* NR29	wild-type group C_1 (6,7)	7
χ3217	*S. montevideo* NR35	wild-type group C_1 (6,7)	7
χ3220	*S. panama* NR38	wild-type group D (1,9.12)	7
χ3246	S. choleraesuis	wild-type group C_1 (6,7)	8
χ3339	S. typhimurium *SL1344*	*hisG46*	9
χ3700	*S. enteritidis* 4973	wild-type group D (1,9,12) PT13A	7
χ3744	*S. typhi* ISP1820	wild-type group D (9,12)	10
χ3761	*S. typhimurium* UK-1	wild-type group B (1,4,12)	11
χ3796	*S. gallinarum*	wild-type group D (1,9,12)	C. Poppe
χ3847	*S. enteritidis* Y-8P2	wild-type group D (1,9,12) PT8	7
χ3848	*S. enteritidis* 27A	wild-type group D (1,9,12) PT8	7
χ3850	*S. enteritidis* B6996	wild-type group D (1,9,12) PT13A	7
χ3851	S. enteritidis	wild-type group D (1,9,12) PT4	Curtiss Collection

Table 1. Bacterial Stains

Strain #	Strain	Phenotype/G notype or	*Reference/ Source*
χ3985	*S. typhimurium* UK-1	*Δcya-12 Δcrp-11*	11
χ4235	*S. kentucky*	wild-type group C_3 (8,20)	Curtiss Collection
χ4433	*S. typhimurium* F98	wild-type group B (1,4,12)	7
χ4860	*S. dublin*	wild-type group D (1,9,12)	C. Maddox
χ4971	*S. typhimurium* UK-1	fur-1	12
χ8387	*S. paratyphi A*	cryptic plasmid cured	ATCC #9281
χ8407	*S. muenster*	wild-type group E_1 (3,10)	Curtiss Collection
χ8409	*S. senftenberg*	wild-type group E_4 (1,3,19)	Curtiss Collection
χ8438	*S. typhi* Ty2	*Cys, rpoS*$^+$ group D (9,12)	13
χ8634	*S. typhimurium* UK-1	ΔPfur223::TT *araC* P_{BAD} *fur*	Curtiss Collection
χ8650	*S. typhimurium* UK-1	*Δpmi-2426*	χ3761
χ8754	*S. typhimurium* UK-1	*Δpmi-2426 Δ*Pfur223::TT *araC* P_{BAD} *fur*	χ8634
χ8600	*S. typhimurium* SL1344	*ΔfliC825 hisG46*	χ3339
χ8601	*S. typhimurium* SL1344	*ΔfljB217 hisG46*	χ3339
χ8602	*S. typhimurium* SL1344	*ΔfliC825 ΔfljB217 hisG46*	
χ8702	*S. typhimurium* SL1344	*ΔmlrA::tetAR*	**14**
χ8844	*S. typhimurium* UK-1	*Δend2311*	χ3761
χ8857	*S. typhimurium* UK-1	*ΔyhiR::TT*	χ3761
χ8865	*S. typhimurium* UK-1	*ΔyhiR::TT Δend2311*	χ3761
χ8874	*S. typhimurium* UK-1	*Δpmi-2426 ΔPfur::araCP*$_{BAD}$*fur ΔfljB217*	χ8754
χ8882	*S. typhimurium* UK-1	*ΔrelA1123*	χ3761

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Table 2. Plasmids

Plasmids	Description	Derivation/source
pBAD/His A, B, and C	N- or C-Terminal 6xHis Tag vector	Invitrogen
pCR-Blunt II	TOPO vector	Invitrogen
pDMS197	SacB suicide vector	Curtiss collection
pRE112	SacB suicide vector	Curtiss collection
pMEG-208	Asd^+ vector with TT *araC* P_{BAD}	Megan Health, Inc
pMEG-375	SacB SacR Pir-dependent suicide vector	Megan Health, Inc
pMEG-855	Suicide vector for ΔPfur223::TT *araC*P_{BAD} *fur*	Megan Health, Inc
pYA3485	Suicide vector for Δ*araE25*	Curtiss collection
pYA3492	Suicide vector for Δ*agfBAC811*	Curtiss collection
pYA3546	Suicide vector for Δ*pmi-2426*	Curtiss collection
pYA3547	Suicide vector for Δ*fliC825*	Curtiss collection
pYA3548	Suicide vector for Δ*fljB217*	Curtiss collection
pYA3582	6xHis tagged FljB	Curtiss collection
pYA3583	6xHis tagged FliC	Curtiss collection
pYA3599	Suicide vector for Δ*araBAD23*	Curtiss collection
pYA3629	Suicide vector for Δ(*gmd-fcl*)-*26*	Curtiss collection

pYA3652	Suicide vector for Δ*endA2311*	Curtiss collection
pYA3654	Suicide vector for Δ*yhiR36*::TT	Curtiss collection
pYA3679	Suicide vector for Δ*relA1123*	Curtiss collection
pYA3686	Suicide vector for Δ*bcsABZC2118*	Curtiss collection
pYA3687	Suicide vector for Δ*bcsEFG2319*	Curtiss collection
pYA3688	Suicide vector for Δ*adrA4118*	Curtiss collection
pYA3701	Suicide vector for Δ*fliC2426*	Curtiss collection
pYA3702	Suicide vector for Δ*fliC*-Var	Curtiss collection

Example 1. Construction of a bacterial strain with arabinose-dependant regulation of the *fur* gene which in turn regulates expression of numerous genes enabling uptake of iron by bacterial cells.

S. typhimurium fur mutants are completely attenuated for mice and chickens but are not very immunogenic. This is undoubtedly due to the fact that *fur* mutants constitutively express a diversity of genes resulting in very efficient uptake of iron that is quite prevalent in the intestinal tract due to dietary non-absorption of iron and due to the presence of iron as a breakdown product of hemoglobin secreted in bile into the duodenal contents of the intestine. Since high intracellular iron concentrations are toxic to bacteria, *fur* mutants do not survive very well in the intestinal tract and therefore are not very efficient in colonization of the GALT, which is necessary in order to be immunogenic. One way to circumvent this problem would be to have the *fur* gene expressed when the bacterial cells are present in the intestinal contents so that efficient colonization of the GALT can take place followed by the gradual cessation in synthesis of the *fur* gene product *in vivo* to result in an attenuated phenotype. In addition, the gradual constitutive expression of *fur* regulated genes would expose the immunized animal host to over expression of iron regulated outer membrane protein (IROMP) antigens as well as other proteins involved in the acquisition, transport and delivery of iron to the bacterial cells. Since many *fur* regulated gene products are closely related structurally among Gram-negative bacterial species, antibodies induced in an immunized animal host to the IROMPs and other *fur* regulated gene products of one bacterial species react with the homologous proteins expressed by other Gram-

negative bacterial pathogens. It should be emphasized that synthesis of *fur* regulated gene products *in vivo* is essential for virulence since a major host defense mechanism is to sequester iron via transferrin, lactoferrin and other iron binding proteins so as to make iron unavailable to invading bacterial pathogens. Thus, antibody responses to these proteins are often protective in preventing successful infection of bacterial pathogens that succeed by *in vivo* multiplication. A corollary is that induction of high-level immune responses to the IROMPs and other *fur* regulated gene products is quite effective in inducing antibodies that are cross protective and prevent infection of an immunized animal host by a diversity of Gram-negative bacterial pathogens.

One means to achieve regulated expression of the *fur* gene is to replace the promoter for the *fur* gene, whose function is regulated by both iron concentration and glucose concentration via the process of catabolite repression, with a metabolically controlled promoter such as that of the arabinose operon. The *araC* P_{BAD} activator-promoter is dependent on the presence of arabinose that binds to the *araC* gene product to activate transcription from the P_{BAD} promoter. Thus, if the *araC* P_{BAD} activator-promoter is used to replace the *fur* promoter and the structural gene for the *fur* gene left intact, expression of the *fur* gene will be dependent on the presence or absence of arabinose. Since arabinose is quite prevalent in plants, some free arabinose exists in the diets consumed by many animals and humans thus contributing to the continued expression of a *fur* gene operationally linked to the *araC* P_{BAD} activator-promoter while bacteria remain in the intestinal tract. On the other hand, arabinose is absent in animal tissues and the *fur* gene product will cease to be synthesized and will thus be diluted out as a consequence of bacterial cell division. Thus, after several cell divisions, constitutive expression of *fur* regulated genes will commence leading to attenuation, on the one hand, and exposure of the immunized animal host to all the *fur* regulated protein antigens, on the other.

To achieve these objectives, primers 1 (SEQ ID NO:1) and 2 (SEQ ID NO:2) (Figure 1-A) were used to PCR amplify a 545 bp fragment from the chromosome of *S. typhimurium* UK-1 χ3761 containing 321 bp upstream of the *fur* gene and 224 bp of the *fur* gene. This blunt-ended PCR amplified DNA fragment was cloned by blunt-end ligation into the pCR-

BluntII-TOPO vector (Figure 1-A, Table 2) which is designed to facilitate blunt-end ligation. The resulting plasmid pMEG-840 (Figure 1-A) was subjected to an inverse PCR reaction using primers 3 (SEQ ID NO:3) and 4 (SEQ ID NO:4) (Figure 1-A) to delete 140 bp containing the *fur* gene promoter from 161 to 22 bp upstream of the *fur* gene ATG start site. The product of this reaction was subjected to blunt-end ligation to yield pMEG-853 (Figure 1-A). The ΔPfur mutation of 140 bp possessed internal restriction sites for *Bgl*II and *Nhe*I separated by 4 bp that would permit insertion of the *araC* P_{BAD} activator-promoter. pMEG-853 was digested with *Spe*I and *Eco*RV and the 472 bp fragment containing the ΔPfur mutation was cloned into the suicide vector pRE112 (Figure 1-A; Table 2) that had been digested with *Xba*I and *Sma*I enzymes to yield pMEG-854 (Figure 1-A; 1-B). It should be noted that the restriction enzymes *Spe*I and *Xba*I generate the same CTAG internal overlapping sticky ends and both *Eco*RV and *Sma*I generate blunt ended sequences to enable success in the cloning and ligation of the 472 bp sequence from pMEG-853 cloned into pRE112 to yield pMEG-854. pMEG-854 contains a 405 bp fragment containing a sequence upstream of the *fur* gene promoter fused to a sequence encompassing the Shine-Dalgarno sequence and beginning of the *fur* gene, which thus contains the ΔPfur mutation. Oligonucleotide primers 5 (SEQ ID NO:5) and 6 (SEQ ID NO:6) (Figure 1-B) were used to PCR amplify the sequence from pMEG-208 (Figure 1-B) containing a transcription terminator (TT) and the *araC* P_{BAD} activator-promoter. This DNA fragment contains a *Bgl*II site and an *Xba*I site encoded in primer 6 (see Figure 1). Since the *Xba*I site generates a CTAG overhang, it is hybridizable with DNA fragments cut with the *Nhe*I restriction enzyme that also generates a CTAG hybridizable sequence. The PCR amplified TT *araC* P_{BAD} fragment from pMEG-208 was therefore digested with *Bgl*ll and *Xba*I and cloned into pMEG-854 digested with *Bgl*II and *Nhe*I to yield the suicide vector pMEG-855 (Figure 1-B).

pMEG-855 was transferred to the suicide vector donor strain MGN-617 (Table 1) that was mated with χ3761 (Table 1). Chloramphenicol-resistant transconjugants that had inherited the suicide vector into the chromosome by a single crossover event were selected by plating on L agar containing chloramphenicol. Ten recombinant colonies were selected and purified on L agar medium with chloramphenicol and individual colonies picked into 1.0 ml of L broth lacking chloramphenicol and incubated at 37°C. Following growth to approximately 10^8

CFU, sucrose-resistant isolates were obtained by plating on CAS plates containing 5 % sucrose but lacking arabinose. This procedure is selective for a second crossover event in which the wild-type *fur* promoter would be replaced with the TT *araC* P_{BAD} activator-promoter that would cause *fur* gene expression to be dependent on the presence of arabinose. Colonies containing cells lacking the ability to synthesize the *fur* gene product have a 3 to 4 mm orange halo surrounding colonies whereas this orange halo is only 1 mm when cells are plated on CAS medium containing 0.2% arabinose. The ΔPfur223::TT *araC* P_{BAD} *fur* construction present in the stocked strain χ8634 is diagramed in Figure 2.

Example 2. Generation of a defined deletion mutation in the *pmi* gene and construction of *Salmonella typhimurium* mutants with this Δ*pmi-2426* mutation.

An 1881 bp *S. typhimurium* DNA sequence encompassing the *pmi* gene was PCR amplified from the S. typhimurium UK-1 χ3761 chromosome. As depicted in Figure 3, oligonucleotide primers 7 (SEQ ID NO:7) and 8 (SEQ ID NO:8) were designed to amplify the 298 bp sequence 5′ to the ATG start codon of the *pmi* gene to yield the N-flanking fragment. Similarly, oligonucleotide primers 9 (SEQ ID NO:9) and 10 (SEQ ID NO:10) were designed to amplify the 301 bp sequence 3′ to the TAG stop codon of the *pmi* gene to yield the C-flanking fragment. The N-flanking and C-flanking DNA fragments (Figure 3) were then digested with *Eco*RI, ligated with polynucleotide joining enzyme after which oligonucleotide primers 7 and 10 were used to amplify the ligated N-flanking and C-flanking fragments by PCR. The PCR amplified oligonucleotide was then digested to completion with *Kpn*I and *Sac*I and cloned into the suicide vector pMDS197 (Table 2) similarly digested with *Kpn*I and *Sac*I. The resulting recombinant suicide vector, pY3546, is depicted in Figure 3. This suicide vector contains the N-flanking and C-flanking sequences adjacent to the *pmi* gene, which has been deleted with the 1176 base pair *pmi* gene replaced with an *Eco*RI recognition sequence.

The suicide vector pYA3546 was introduced by electroporation into the suicide vector donor strain MGN-617 (Table 1). This recombinant strain was then mated with the *S. typhimurium* UK-1 strain χ3761 (Table 1) and tetracycline-resistant transconjugants were

selected that arose due to single cross over events integrating pYA3546 into the chromosome of χ3761. Ten tetracycline-resistant transconjugants were selected, purified by restreaking on tetracycline-containing medium and grown in tetracycline-free Luria broth as 1 ml cultures to an approximate density of 10^8 CFU/ml. These cultures were plated in the presence of 5% sucrose to select for a second crossover event to excise the suicide vector from the chromosome but leave in its place the deletion of 1176 bp encoding the *pmi* gene. Individual isolates were tested for inability to ferment mannose on MacConkey-Mannose agar and one isolate designated χ8650 was stocked and the *pmi* allele designated *pmi-2426*. The chromosomal Δ*pmi-2426* mutation present in χ8650 is diagramed in Figure 4 along with the genes flanking the deleted *pmi* mutation in the *S. typhimurium* chromosome.

Example 3. Introduction of Δ*pmi-2426* mutation into χ8634.

The suicide vector pYA3546 (Figure 3) for introduction of the Δ*pmi-2426* mutation by allele replacement was introduced into MGN-617 (Table 1) and this strain mated with χ8634 possessing the ΔPfur223::TT *araC* P_{BAD}*fur* mutation. Tetracycline-resistant transconjugants were selected on L agar medium containing tetracycline and 0.2% arabinose. It should be noted, that strains with the ΔPfur223::TT *araC* P_{BAD}*fur* mutation grow rather poorly on medium without any added arabinose. Ten tetracycline-resistant transconjugants were purified by restreaking on L agar medium containing tetracycline and 0.2% arabinose. Individual colonies were picked into 1.0 ml of L broth containing 0.2% arabinose. When cultures reached approximately 1 x 10^8 CFU, sucrose-resistant isolates, in which a second crossover event had occurred, were selected by plating on L agar medium containing 5% sucrose and 0.2% arabinose. Sucrose-resistant isolates were picked and tested for sensitivity to tetracycline indicating loss of the suicide vector and for inability to ferment mannose by streaking on MacConkey-Mannose agar. One isolate having all of the correct phenotypic properties with regard to the presence of the Δ*pmi-2426* and ΔPfur223::TT *araC* P_{BAD}*fur* mutations was stocked as χ8754.

Example 4. Phenotypic properties of χ8634, χ8650 and χ8754.

χ8634 with the ΔPfur223::TT *araC* P_{BAD}*fur* mutation, χ8650 with the Δ*pmi-2426* mutation and χ8754 with both mutations were compared to the wild-type *S. typhimurium* UK-1 strain χ3761 for ability to ferment various carbohydrates contained at a 0.5% concentration in MacConkey agar. As indicated by the data in Table 3, all strains are unable to ferment lactose whereas χ8650 and χ8754 are unable to ferment mannose. All other sugars were fermented by all four strains.

Table 3. Carbohydrate fermentations[a]

Strains/genotype	Carbohydrates							
	Lac	Glc	Man	Mal	Srl	Xyl	Ara	Fru
χ3761 wild-type	-	+	+	+	+	+	+	+
χ8634 ΔPfur223::TT *araC* P_{BAD}*fur*	-	+	+	+	+	+	+	+
χ8650 Δ*pmi-2426*	-	+	-	+	+	+	+	+
χ8754 Δ*pmi-2426* Δfur223::TT *araC* P_{BAD}*fur*	-	+	-	+	+	+	+	+

[a] Bacterial strains were grown in L broth at 37°C overnight and the cultures streaked to observe isolated colonies on MacConkey agar with 0.5% each of the sugars indicated. Plates were incubated overnight. Lac, lactose; Glc, glucose; Man, mannose; Mal, maltose; Srl, sorbitol; Xyl, xylose; Ara, arabinose; Fru, fructose; -, no fermentation; +, fermentation.

The same four strains were evaluated for production of the group B LPS O-antigen side chains and for presence of flagellar antigens using slide agglutination assays with antisera obtained from Difco Laboratories. The results presented in Table 4 are as expected. It should be noted that L agar, which contains yeast extract, contains a low concentration of mannose. Thus strains with the Δ*pmi-2426* mutation when grown in L broth or on L agar make a lower than usual level of O-antigen side chains than if grown in medium with added mannose but a higher amount than when grown in a medium totally devoid of mannose. For example, if the strains are grown in Nutrient broth or on Nutrient agar medium without added mannose, the amount of O-antigen side

chains synthesized is very negligible as revealed by resistance of the strains to infection with bacteriophage P22 whose attachment to *S. typhimurium* is dependent on the presence of O-antigen side chains.

Table 4. Slide agglutination assays with *Salmonella* O and H anti-sera[a]

Strains/genotype	Group B O antiserum factors 1, 4, 5,12	H antiserum polyA
χ3761 wild-type	+++	+++
χ8634 ΔPfur223::TT *araC* P_{BAD}*fur*	+++	+++
χ8650 Δ*pmi-2426*	++	+++
χ8754 Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur*	++	+++

[a] Bacterial strains were grown on L agar without mannose and arabinose. A single colony of each of the strains was picked and suspended in buffered saline with gelatin (BSG) on a microscope slide, and mixed with 5 µl of the anti-serum. Agglutination reactions were observed and compared. ++- moderate agglutination; +++- high agglutination.

Figure 5 presents the results of an experiment with χ8650 with the Δ*pmi-2426* mutation, which demonstrates that as a function of time or number of generations of growth in Nutrient broth medium in the absence of added mannose there is a gradual loss of LPS O-antigen side chains. This behavior is as expected and would be reproduced in vivo when a vaccine strain, after immunization of an animal host, enters visceral tissues which lack free non-phosphorylated mannose.

Based on the nature of mutational changes in χ8634 and χ8754, which both possess the ΔPfur223::*araC* P_{BAD}*fur* mutation, synthesis of IROMPs should be constitutive when those strains are grown in the absence of arabinose and absent when grown in the presence of arabinose. The synthesis of IROMPs should be unaffected by the presence or absence of arabinose during growth of χ3761 with the level of IROMPs dependant on the iron concentration

in Nutrient broth. These predictions were evaluated by preparing overnight cultures of χ3761, χ8634 and χ8754 growing statically in 10 ml of Nutrient broth containing 0.2% arabinose at 37°C. The cultures were then diluted 1:1000 into 10 ml of prewarmed Nutrient broth with and without 0.2% arabinose and grown with aeration to a cell density of about 8 x 10^8 CFU/ml. The cultures were centrifuged at 5000 rpm at 4°C for 15 min in a refrigerated Sorvall clinical centrifuge and the cell pellets suspended in 10 mM HEPES buffer. The bacterial suspensions were lysed by sonication with six 10 s pulses at 40 w. The sonicated suspensions were centrifuged at 15,600 rpm for 2 min at 4°C. The supernatant fluid was centrifuged again at 15,600 rpm for 30 min at 4°C. The cell membrane pellets were suspended in HEPES buffer and an equal volume of 2 % Sarkosyl added. The suspension was incubated at room temperature for 30 min with gentile shaking. Next, the outer membrane aggregate was sedimented by centrifugation at 15,600 rpm for 30 min at 4°C and the supernatant was discarded. The membrane pellets were washed with and re-suspended in HEPES buffer. The samples were prepared for the SDS-PAGE analysis by adding equal amounts of 2X sample buffer and boiling the samples for 10 min. Lastly, the samples were centrifuged at 12,000 rpm for I min in a microfuge and loaded onto gels containing SDS and 10 % polyacrylamide. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue. The results are depicted in Figure 6 and give the expected results based on the strain genotypes.

Example 5. Ability of mutant strains to colonize lymphoid tissues in mice.

The ability of *S. typhimurium* χ8634 with the ΔPfur223::*araC* P_{BAD} *fur* mutation to colonize eight-week-old female BALB/c mice following oral inoculation of 10^9 CFU was investigated. The bacteria were grown in Luria broth containing 0.2% arabinose to an OD_{600} of approximately 0.8. Bacteria were sedimented by centrifugation and concentrated by suspension in buffered saline with gelatin (BSG) so that 20 µl would contain approximately 10^9 CFU of bacteria. Groups of immunized mice were euthanized as a function of time after oral inoculation and the data pertaining to colonization of Peyer's patches and spleens are depicted in Figure 7. It is evident that χ8634 is quite effective in colonization of lymphoid tissues whereas a strain with a deletion of the *fur* gene colonizes tissues at very much lower titers such that animals do not

develop immunity to subsequent challenge with virulent wild-type *S. typhimurium*. Results from an experiment done the same way for the *S. typhimurium* strain χ8650 with the Δ*pmi-2426* mutation are presented in Figure 8. In this case, bacteria were grown in Luria-Bertani broth with or without 0.5% mannose prior to inoculation into mice. There were no significant differences for the two growth conditions.

Results of two other experiments with the *S. typhimurium* χ8754 strain that possesses both the ΔPfur223::TT *araC* P_{BAD} *fur* and Δ*pmi-2426* mutations are represented in Figures 9 and 10. It is evident that χ8754 persists for a sufficient time in lymphoid tissues to induce immunity before almost disappearing by 42 days (Figure 9). Results were not significantly different depending upon whether the cultures were grown in the presence or absence of mannose and arabinose prior to inoculation (Figure 10). This result is anticipated in that Luria broth, as indicated above, contains yeast extract that possesses both free arabinose and free mannose at low concentrations. When strains are grown in Nutrient broth, the differences are magnified but growth of *Salmonella* vaccine strains in Nutrient broth leads to a lesser degree of colonization and a lower immunogenicity. Growth in Nutrient broth is thus not a preferred method of evaluation for attenuated live vaccines.

Example 6. Avirulence and immunogenicity of *S. typhimurium* strains with Δ*pmi-2426* and/or ΔPfur223::TT *araC* P_{BAD} *fur* mutations.

Table 5 presents results of an experiment to evaluate the attenuation and immunogenicity of χ8634 with the ΔPfur223::TT *araC* P_{BAD} *fur* mutation. χ8634 was grown in Luria broth either without or with 0.2% arabinose to an OD_{600} of about 0.8. Bacterial cells were sedimented by centrifugation and suspended in BSG to a density so that there would be about 1 x 10^9 CFU in a 20 μl sample. Female BALB/c mice were purchased at 7 weeks of age and maintained for one week in our animal facilities to acclimate prior to use in experiments. At eight weeks of age, food and water were removed for four hours prior to oral inoculation with 20 μl of χ8634 cells suspended in BSG at appropriate densities. Morbidity and mortality were observed for 30 days, after which, survivors were challenged with virulent wild-type *S.*

typhimurium UK-1 χ3761 grown in Luria broth to an OD_{600} of approximately 0.8. It is apparent from the results that growth in Luria broth without added arabinose conferred total avirulence and induced the highest level of protective immunity. Since Luria broth contains yeast extract, which contains arabinose, it is evident that addition of an extra 0.2% arabinose must cause synthesis of too much Fur protein such that the total repression of all *fur*-regulated genes must starve cells for iron so that they are less able to survive and colonize in the intestine and thus are less immunogenic. This result has been observed in other experiments and thus growth of strains in Luria broth without added arabinose will be preferred to optimize immunogenicity. If, on the other hand, χ8634 is grown in Nutrient broth, which lacks arabinose, the addition of arabinose to 0.1 or 0.2% is necessary to achieve good immunogenicity.

Table 5. Virulence and protection of *S. typhimurium* UK-1 ΔPfur223::TT *araC* P_{BAD}*fur* mutant χ8634 in 8-week-old female BALB/c mice following oral inoculation[a]

Growth condition	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge
Luria broth	1.4×10^9	4/4	1.4×10^9	4/4
	1.4×10^8	4/4	1.4×10^9	4/4
	1.4×10^7	4/4	1.4×10^9	4/4
	1.4×10^6	4/4	1.4×10^9	3/4
	1.4×10^5	4/4	1.4×10^9	2/4
(Total)		20/20		17/20
Luria broth with 0.2% arabinose	1.1×10^9	4/4	1.4×10^9	4/4
	1.1×10^7	3/4	1.4×10^9	2/3
	1.1×10^6	4/4	1.4×10^9	1/4
	1.1×10^5	4/4	1.4×10^9	0/4
(Total)		15/16		7/15

[a] Bacteria were grown in Luria broth with or without 0.2% arabinose to OD_{600} of ~0.8. Bacterial cells were collected by centrifugation and suspended in buffered saline with

gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with virulent wild-type UK-1 χ3761 grown in Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

To evaluate the attenuation and immunogenicity of *S. typhimurium* χ8650 possessing the *Δpmi-2426* mutation, bacteria were grown in Nutrient broth with or without 0.5% mannose and 0.5% glucose to an OD_{600} of approximately 0.8. Bacterial cells were collected by centrifugation and suspended in a concentrated form in BSG so that a 20 µl sample would possess approximately 1 x 10^9 CFU. Female BALB/c mice were purchased at 7 weeks of age and maintained for one week in our animal facilities to acclimate prior to use in experiments. At eight weeks of age, food and water were removed for four hours prior to oral inoculation with χ8650 cells suspended in BSG at appropriate densities. Morbidity and mortality were observed for 30 days, after which, survivors were challenged with virulent wild-type *S. typhimurium* UK-1 χ3761 grown in Luria broth to an OD_{600} of approximately 0.8. It should be noted that the vaccine strain was grown in Nutrient broth since it is almost devoid of mannose to determine the influence of O-antigen side chain synthesis on the initial invasiveness of the candidate vaccine strain. On the other hand, we have demonstrated in many past studies that growth in Luria broth leads to optimal expression of the phenotype that is conducive to attachment to and invasion into the GALT of both virulent as well as of attenuated *Salmonella* vaccine strains. The results of this experiment are presented in Table 6. It is evident that growth of the vaccine strain under conditions that enable synthesis of LPS O-antigen side chains leads to morbidity and mortality at high doses (i.e., 1.5 x 10^9 CFU). However, mice that survived these high doses without morbidity, acquired protective immunity to high doses of the challenge strain. χ8650 grown in medium to preclude synthesis of LPS O-antigen side chains were totally attenuated and induced a high level of protective immunity (Table 6).

Table 6. Virulence and protection of *S. typhimurium* UK-1 *Δpmi-2426* mutant χ8650 in 8-week-old female BALB/c mice following oral inoculation[a]

Growth condition	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge
Nutrient Broth + 0.5% Man + 0.5% Glc	1.5×10^9	3/8	8.0×10^8	3/3
	1.5×10^8	7/8[b]	8.0×10^8	4/4
	1.5×10^7	7/8	8.0×10^8	3/4
	8.0 x 107	3/3		
	1.5×10^6	4/4	8.0×10^7	4/4
	1.5×10^5	4/4	8.0×10^7	4/4
		(25/32)		**(21/22)**
Nutrient Broth:	1.7×10^9	8/8	8.0×10^8	4/4
	8.0×10^7	4/4		
	1.7×10^8	8/8	8.0×10^8	4/4
	8.0×10^7	4/4		
	1.7×10^7	7/8	8.0×10^8	3/3
	8.0×10^7	4/4		
	1.7×10^6	4/4	8.0×10^7	4/4
	1.7×10^5	4/4	8.0×10^7	2/4
		(31/32)		**(28/31)**

[a] Bacteria were grown in Nutrient broth with or without 0.5% mannose and 0.5% glucose to OD_{600} of ~0.8. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with virulent wild-type UK-1 χ3761 grown in Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

[b] Three of the seven surviving mice (in one cage) appeared sick with loss of hair and were therefore not challenged.

We next investigated the attenuation and immunogenicity of χ8754, which possesses both the ΔPfur223::TT *araC* P_{BAD}*fur* and Δ*pmi-2426* mutations. χ8754 was grown in Luria broth supplemented with 0.5% mannose and 0.2% arabinose to an OD_{600} of approximately 0.8. Bacterial cells were concentrated by centrifugation and suspended in BSG such that a 20 μl inoculum would contain approximately 1 x 10^9 CFU. Eight-week-old female BALB/c mice that had been acclimated for a week were orally inoculated with 20 μl of inocula containing differing densities of χ8754 cells. All mice survived for 30 days as indicated by the results presented in Table 7. The surviving mice were challenged with 1.0 x 10^9 CFU of the wild-type virulent *S. typhimurium* UK-1 strain χ3761 and all but one mouse survived the challenge. In that we had found that χ8634 with the ΔPfur223::TT *araC* P_{BAD}*fur* mutation displayed total attenuation and highest immunogenicity when grown in Luria broth lacking added arabinose and since we had observed less morbidity and mortality when χ8650 with the Δ*pmi-2426* mutation was grown in Luria broth without added mannose, it has become our practice to grow the doubly mutant strain in Luria broth without added mannose or arabinose. These growth conditions yield total attenuation to inoculation with high titers of the vaccine strain and induce the highest level of protective immunity to challenge with wild-type *S. typhimurium*.

Table 7. Virulence and protection of *S. typhimurium* UK-1 Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur* mutant χ8754 in 8-week-old female BALB/c mice following oral inoculation[a]

Strain	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge
χ3761 wild-type			1.0 X 10^7	0/5
χ8754 Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur*	1.1 x 10^9	5/5	1.0 x 10^9	5/5
	1.1 x 10^8	5/5	1.0 x 10^9	5/5
	1.1 x 10^7	5/5	1.0 x 10^9	4/5

[a] Bacteria were grown in Luria broth supplemented with 0.5% mannose and 0.2% arabinose to OD_{600} of ~0.8. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with virulent wild-type UK-1 χ3761 grown in Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

Example 7. Induction of cross protective immunity to challenge with wild-type *S. enteritidis*.

Eight-week-old female BALB/c mice were orally inoculated with decreasing doses of χ8754 grown in Luria broth (without added mannose or arabinose) to an OD_{600} of approximately 0.8 and suspended in BSG. In this experiment, immunized mice were challenged 30 days later with *S. enteriditis* strain χ3700 (phage type 13a) also grown in Luria broth to an OD_{600} of approximately 0.8 and resuspended in BSG. Eighty percent of mice immunized with either the highest dose of χ8754 or with a dose of χ8754 that was 10-times less than the challenge dose of χ3700, survived challenge with χ3700 (Table 8). Mice immunized with a vaccine inoculum only 1% of the challenge inoculum were not protected (Table 8). It is therefore evident that there is a significant level of cross protective immunity induced by the group B *S. typhimurium* ΔPfur223::TT *araC* P_{BAD}*fur* Δ*pmi-2426* candidate vaccine strain to challenge with a wild-type group D *S. enteriditis* strain known to be capable of egg-transmitted disease in humans. Based on past results, it would be expected that the level of cross protective immunity would be further enhanced by a booster immunization seven or so days after the initial immunization.

Table 8. Cross protection in mice immunized with *S. typhimurium UK-1* Δ*pmi-2426* ΔPfur223::TT *araC* P_{BAD}*fur* strain □8754 and challenged with *S. enteritidis* wild-type χ3700[a]

Strain	Inoculating dose	Survivors / total	Challenge dose	Survivors /total after challenge	MDD[b]
χ3700			1.2 X 10^9	0/5	wild-type
χ8754 *Δpmi-2426*	1.0 x 10^9	5/5	1.2 x 10^9	4/5	12
ΔPfur223:TT *araC*P_{BAD}*fur*	1.0 x 10^8	5/5	1.2 x 10^9	4/5	14
	1.0 x 10^7	5/5	1.2 x 10^9	0/5	10.5

[a] Bacteria were grown in Luria broth to OD_{600} of ~0. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with wild-type *S. enteritidis* χ3700 grown in Luria broth. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

[b] MDD: Mean day of death.

Example 8. Induction of serum antibody responses against OMPs and IROMPs in diverse serotypes of *Salmonella* and in several strains of *E. coli*.

Serum antibodies were collected 30 days after oral inoculation of mice with either χ8650 with the Δ*pmi-2426* mutation or χ8634 with the ΔPfur223::TT *araC* P_{BAD} *fur* mutation by retro orbital bleeding. Serum IgG antibodies to *Salmonella* and *E. coli* OMPs and IROMPs were quantitated by ELISA. Briefly, 96-well ELISA plates were coated with OMPs or IROMPs isolated from *Salmonella* and *E. coli* strains (see below). The plates were blocked with 1 % BSA in PBS plus 0.1 % Tween 20 (blocking buffer). Serum samples were pooled from 4 mice and diluted 1:400 in blocking buffer. A volume on 100 µl of each diluted sample was added in

duplicate to the 96-well plates, incubated at 37°C for 2 h and washed with PBS plus 0.05 % Tween 20. The plates were then incubated with biotin-avidin-labeled goat anti-mouse IgG (1:1000 in blocking buffer) and alkaline phosphatase-labeled Extravidin (1:4000 in blocking buffer). *p*-nitrophenylphosphate (1 mg/ml) in 0.1 M diethanolamine buffer was used as a substrate. The absorbency of the color reaction was read at 405 nm with an automated ELISA reader.

The OMPs and IROMPs as the test antigens for ELISA were isolated from bacteria of various serotypes of *Salmonella* and *E. coli* (Table 1). The bacteria were grown in Luria broth plus 200 mM $FeCl_3$ to repress synthesis of IROMPs and in Luria broth plus 200 mM α,α'-dipyridyl to sequester iron and cause IROMP synthesis to be constitutive. Bacterial cells were collected by centrifugation and the cell pellets suspended in 10 mM HEPES buffer. The cell suspension was sonicated with six 10 s pulses at 40 w. The sonicated suspension was centrifuged at 15,600 x g for 2 min at 4°C. The supernatant fluid was centrifuged again for 30 min at 4°C. The cell membrane pellets were suspended in HEPES buffer and an equal volume of 2 % Sarkosyl added. The suspension was incubated at room temperature for 30 min with gentile shaking. The suspension was then centrifuged at 15,600 x g for 30 min and the supernatant was discarded. The membrane pellets were washed with and re-suspended in HEPES buffer. The concentration of protein in each preparation was determined. Separate ELISA plates were coated with OMP and IROMP preparations (200 ng/well) from each strain used in the analysis. It should be noted that the IROMP preparations also contain OMPs.

It is evident from the data presented in Figure 11 that both bacterial vaccines induced significant titers of antibodies that react with the OMPs present in serogroups C1, C2, C3, D and E1. In addition, significant antibody titers were induced to the OMPs of most of the *E. coli* strains with the lowest titers to the OMPs present in the totally attenuated laboratory *E. coli* K-12 strain χ289 (Figure 11).

The same serum antibodies were used to determine the antibody titers against IROMPs obtained from the same bacterial strains used in the proceeding experiment. As

revealed by the data in Figure 12, both χ8650 and χ8634 induced substantial antibody responses to the IROMPs from all strains of *Salmonella* and *E. coli* evaluated. The results of these two experiments are in accord with the evidence for cross protective immunity as revealed by challenge of immunized mice with a heterologous *S. enteriditis* group D strain (Table 8).

Example 9. Attenuation of *S. typhimurium* strains with Δ*pmi-2426* and ΔPfur::TT *araC* P_{BAD} *fur* in day-of-hatch white leghorn chicks.

Results presented in Table 9 indicate that *S. typhimurium* strain χ8754 is completely attenuated when used to inoculate day-of-hatch chicks at doses in excess of 1 x 10^9 CFU. For these experiments, the day-of-hatch chicks were infected before being provided with either food or water. These white leghorn chicks are hatched in our animal facility from fertile eggs obtained from SPAFAS. Bacteria for infection are grown in Luria broth and concentrated in BSG in the same manner as used for experiments to infect mice as described above. In this experiment, the LD_{50} for χ8754 was in excess of 4 X 10^9 (Table 9). The same result was observed with χ8754 grown in Luria broth without added mannose and arabinose (data not shown). However, some chicks survived infection with 1 X 10^7 CFU of the wild-type χ3761, a dose that is far in excess of the LD_{50}. This result is sometimes observed due to a very rapid stimulation of a protective innate immune response by the high inoculating dose of virulent bacteria. This type of response is seen more often in birds that are naturally more refractory to infection by *Salmonella* than in inbred mice. Results are also more variable since the chickens are out bred and we do not get fertile eggs from the same flock of breeders for each shipment from SPAFAS.

Table 9. Virulence of S. typhimurium UK-1 Δ*pmi-2426* ΔPfur223::TT*araC* P_{BAD} *fur* mutant χ8754 in day-of-hatch chicks following oral inoculation

Strains/Genotype	Inoculation Dose (cfu)	Survivors/total	LD50
χ8754/ΔPfur::araC	4.3 x 10^9	4/4	> 4 X 10^9

Table 9. Virulence of S. typhimurium UK-1 Δ*pmi-2426* ΔPfur223::TT*araC* P_{BAD} *fur* mutant χ8754 in day-of-hatch chicks following oral inoculation

Strains/Genotype	Inoculation Dose (cfu)	Survivors/total	LD50
PBADfur11			
	2.3×10^9	4/4	
	1.3×10^9	4/4	
χ3761/wild-type	1.2×10^7	2/4	

Example 10. Ability of candidate vaccine strains to colonize and persist in lymphoid tissues of vaccinated chicks.

Day-of-hatch chicks were orally inoculated with the candidate vaccine strain χ8754 grown in L broth to an OD_{600} of 0.8 and suspended in BSG. Groups of chicks were euthanized on various days after initial infection to quantitate the titers of χ8754 in the bursa of Fabricius, the spleen and in cecal contents. Results of these studies are presented in Figure 13. The increases in titers at 28 days after inoculation were unusual and unexpected. However, in the evaluation of the ability of χ8754 to colonize mice, the titers dropped significantly after 28 days (Figure 9).

Example 11. Introduction of Δ*fliC825* and Δ*fljB217* mutations into the candidate vaccine strain χ8754.

The various *Salmonella* serotypes generally have genetic information to express two antigenically different flagellar antigens (a minority express only one) and employ a genetic switching mechanism for phase variation to express one or the other flagellar antigenic type. Since the flagellar antigens are very immunogenetic and since there is great diversity of antigenic flagellar types in enteric bacteria infecting the intestinal tract that do not exhibit a significant degree of antigenic similarity, we have deleted the genes for the *S. typhimurium fliC* and *fljB* flagellar antigens. This decision was based on the fact that antibodies to the FliC and FljB

flagellar antigens would not be of significance in inducing cross protective immunity and that induction of immune responses to these antigens would compete with the induction of antibody responses to the common LPS core antigen or to the highly cross reactive OMP and IROMP surface protein antigens that are important for induction of cross protective immunity. The construction of the suicide vector pYA3547 for introduction of the Δ*fliC825* mutation into the chromosome is shown in Figure 14. The construction of the suicide vector pYA3548 for introduction of the Δ*fljB217* mutation into the chromosome is shown in Figure 15. The molecular genetic attributes of the Δ*fliC825* and Δ*fljB217* mutations upon introduction into the chromosome are depicted in Figure 16. Both of these suicide vectors are transferred to MGN-617 (Table 1) and the constructed strains used for conjugational transfer of the suicide vectors to χ8754 possessing the Δ*pmi-2426* and ΔPfur::TT *araC* P_{BAD} *fur* mutations. In the first step, transfer by MGN-617 of pYA3547 to χ8754 followed by selection for chloramphenicol resistance yields recombinants with the suicide vector integrated into the chromosome. These chloramphenicol-resistant recombinants are then grown in L broth in the absence of chloramphenicol and subjected to selection for sucrose-resistant isolates by plating on L agar containing 5 % sucrose. This selection results in loss of the integrated suicide vector by a second reciprocal crossing over event to often result in allele replacement with inheritance of the Δ*fliC825* mutation in place of the wild-type allele. The Δ*flgB217* allele is introduced in the same way starting with the transfer by MGN-617 of the suicide vector pYA3548 and its subsequent integration (by selecting for tetracycline resistance) into and then excision (by selecting for sucrose resistance) from the chromosome for allele replacement. This generated the *S. typhimurium* strain χ8874 (Table 1) that possesses the Δ*flgB217* mutation in addition to the mutations present in the χ8854 parent (Table 1). Following construction, strains are evaluated to demonstrate the absence of motility and the absence of flagellar antigens by a negative slide agglutination test with the Difco antisera against *Salmonella* flagellar antigens used previously (see Example 4). The presence of all four mutational alterations can be validated by PCR analyses and conduct of tests for the phenotype associated with each mutation as described in previous examples.

Example 12. Evaluation of induction of cross protective immunity in chickens.

Experiments to evaluate induction of cross protective immunity against diverse *Salmonella* serotypes is by a slight modification of the methods worked out and described by Hassan and Curtiss (1994, Infect, Immun. 62:5519-5527). Day-of-hatch chicks are immunized orally with 10^8 CFU of the vaccine described in Example 11 above with a booster immunization of the same dose administered 10 days later. These chicks and groups of unimmunized chicks as controls are challenged with *Salmonella* of numerous serotypes as listed in Table 1. Vaccine and challenge strains are grown in Luria broth and resuspended in BSG before oral inoculation. Groups of five challenged birds are euthanized 7 and 14 days after challenge and the titers of the challenge strain in the bursa of Fabricius, spleen, liver, ovaries and in the contents of the small intestine (ileum) and cecum determined. To evaluate induction of cross protective immunity against APEC infection, the APEC challenge strains can be administered by injection into the caudal air sac or by intratracheal inoculation.

Example 13. Construction of mutant derivatives of host-specific *Salmonella* serotypes for use as vaccines to induce cross protective immunity to gram-negative enteric pathogens in swine, cattle and humans.

S. choleraesuis is a host-adapted *Salmonella* that predominantly infects swine. *S. dublin* is a host-adapted *Salmonella* that predominately infects cattle. *S. paratyphi* A and *S. typhi* are host-adapted *Salmonella* that predominantly infect humans. The suicide vectors and methods for introducing the Δ*pmi-2426* and ΔPfur::TT *araC* P_{BAD} *fur* mutations are the same as described in the Examples given above. Each of these *Salmonella* serotypes possesses unique genes for the predominant flagellar antigens. Therefore, specific suicide vectors based on DNA sequence information for the flagellar genes in each of these serotypes is used to generate deletions for both flagellar antigen genes in each of the serotypes. The *S. choleraesuis* χ3246, *S. dublin* χ4860,*S. paratyphi* A χ8387 and *S. typhi* χ3744 and χ8438 strains that are altered by these genetic manipulations are listed in Table 1. The presence of each of the mutations can be ascertained by PCR analyses and testing for the specific phenotype associated with each

mutation. Difco antisera is used to verify the presence of the appropriate group A, C1 or D O-antigens. The *S. choleraesuis* and *S. dublin* vaccines can initially be evaluated for induction of cross protective immunity in mice using challenge of immunized mice with a diversity of *Salmonella* strains of different serotypes (Table 1) as well as with other gram-negative enteropathogens. Subsequent evaluations would use pigs and calves to substantiate induction of cross protective immunity by the candidate *S. choleraesuis* and *S. dublin* vaccines, respectively. The *S. paratyphi* A and *S. typhi* candidate vaccines will be evaluated in human volunteers since there is no suitable animal model.

Example 14. Elimination of serotype-specific flagellar antigens while retaining the flagellar constant domains of FliC that serve as a pathogen-associated molecular pattern (PAMP) to trigger an innate immune response by specific interaction with the toll-like receptor 5 (TLR5).

Although eliminating the ability of vaccine strains designed to induce cross-protective immunity to induce immune responses to serotype-specific flagellar antigens as outlined in Example 11 is logical, these flagellar antigens, especially FliC, contain very strong T-cell epitopes (Cookson and Bevan, 1997, J. Immunol. 158:4310-4319) and thus might be important in inducing cellular immunity against *Salmonella* that would be protective against infection by diverse *Salmonella* serotypes. Potentially more important, flagella on bacteria serve as one of the pathogen-associated molecular patterns (PAMPs) and specifically trigger an innate immune response by their specific interaction with the toll-like receptor 5 (TLR5) (Hayashi et al., 2001, Nature 410:1099-1103). It has recently been determined for the *E. coli* FliC protein that elimination of the central variable serotype-specific domains with retention of the N-terminal and C-terminal α-helical constant domains permits TLR5 recruitment and IL-8 production (Donnelly and Steiner, 2002, J. Biol. Chem. 277:40456-40461). Importantly, the flagellar T-cell epitope is contained within the conserved amino acid sequences of the flagellar antigens (Joys et al., 1993, Infect. Immun. 61:1146-1148; McSorley et al., 2000, J. Immunol. 164:986-993). We have therefore redesigned the deletion mutation for the flagellar *fliC* gene so that the modified *fliC* gene will no longer have any variable domains but will retain the N-terminal and C-terminal constant domains forming a conservative flagellar structure capable of interacting with TLR5 to stimulate the innate immune response and also in inducing cellular immune responses. We will

use this mutation, Δ*fliC*-Var (minus variable region of *fliC* gene), in conjunction with the complete deletion mutation of the *fljB* gene, Δ*fljB217*. Figure 17 diagrams the construction of the suicide vector (listed in Table 2) for delivery into the chromosome of the Δ*fliC*-Var deletion mutation that deletes the variable FliC flagellar amino acid domains. Figure 17 also lists the oligonucleotide primers needed to generate the Δ*fliC*-Var mutation.

As described in Example 10 and diagramed in Figure 14, we had constructed a suicide vector to introduce the Δ*fliC825* mutation into the chromosomes of attenuated *Salmonella* vaccine strains. In this construction, we deleted 1380 bp of the 1488 bp encoding the entire *fliC* gene with short coding sequences for the N-terminal and C-terminal ends of FliC protein remaining. We have therefore constructed the suicide vector (Table 2) for the improved Δ*fliC2426* mutation (Figure 18) that deletes the entire coding sequence of the *fliC* gene. We will hereafter use this Δ*fliC2426* mutation in strains to compare with the Δ*fliC*-Var mutation that retains the PAMP attributes but deletes serotype-specific flagellar antigen domains.

Figure 19 diagrams the chromosomal Δ*fliC*-Var and Δ*fljiBC2426* mutations. (The Δ*fljB217* mutation is diagramed in Figure 16.) These mutations can be transferred to other *Salmonella* vaccine strains being constructed using the methods described by Kang et al (2002, J. Bacteriol. 184:307-312). As listed in Table 1, we have constructed recombinant pBAD/His vectors that generate production of His-tagged FliC and His-tagged FljB proteins and have purified these proteins by standard methods using nickel columns. These purified proteins have been used to generate anti-flagellar antibodies in rabbits that react with intact flagella possessing the serotype-specific antigenic determinants but should fail to interact with flagella that retain the constant domains but lack the variable amino acid sequences necessary for serotype specificity. To further complete this analysis, a His-tagged FliC-Var protein lacking the variable domains will be constructed by PCR cloning of the mutated sequences from the suicide vector diagramed in Figure 17 into the pBAD/His vector (Table 2) and the protein purified to demonstrate that this protein does not significantly react with antibodies raised against the intact FliC protein but is able to interact with Caco-2 cells to elicit production of IL-8 (Donnelly and Steiner, 2002, J. Biol. Chem. 277:40456-40461).

Example 15. Method for assessing induction of antibodies by candidate vaccine constructions that possess the abilities to interact with surface antig ns on *Salmonella enterica* isolates of divers serotyp s and other closely related strains of *Enterobacteriaceae*.

Since quantitative antibody titers against isolated bacterial OMPs and IROMPs could represent antibodies that react, in part, with antigenic determinants that are masked in the intact bacterial cells, such antibody titers might be somewhat misleading as an indication of the ability of candidate vaccines to induce antibodies that would be cross reactive in a protective way against diverse enteric bacteria. For this reason, we have modified and refined for our use a quantitative ELISA to accurately measure antibodies that recognize whole live as well as whole killed bacteria of diverse serotypes and species. (see Mowat and Reed, 1994, *In* Current Protocols in Immunology, Gligan et al., eds., John Wiley and Sons, Inc., pp. 2.0.1-2.11.12; Marcjanna et al., 2001, Vet. Microbiol. 78:61-77). In this modified ELISA method, varying concentrations of washed bacteria (10^5 to 10^9 CFU) are reacted with various dilutions of non-immune (as a control) and immune sera (diluted 1:100 to 1:3,200) in a crisscross serial dilution titration analysis. The *S. enterica* serotypes and *E. coli* strains used to collect the data in Figures 11 and 12 (Example 8) are used in these analyses as well as additional bacterial strains available to us (Table 1). The antibody titer determinations from such studies can be correlated with animal studies to evaluate the ability of candidate vaccines to induce cross protective immunity to viable pathogenic challenge strains. These studies will establish the antibody titers necessary as a correlate of inducing protective immunity and thus will eliminate the need for using vast numbers of animals immunized with candidate vaccines and challenged with a very large diversity of enteric bacterial pathogens. This method that we have developed will permit vaccine evaluation to be more economical and very much reduce the need for extensive animal experimentation, which would also be very costly. In addition to this modified whole-cell ELISA method, with either live or whole killed bacteria serving as antigens, we can also employ indirect immunofluorescence microscopy to determine whether antibodies in sera of animals immunized with candidate vaccines are reactive against surface bacterial antigens as visualized with intact bacteria.

Example 16. Construction of a new ΔPfur::TT *araC* P_{BAD}*fur* deletion-insertion mutation with tighter *araC* P_{BAD} regulation for use in *S. paratyphi* A and *S. typhi* vaccine constructions.

Vaccine strains with ΔP_{fur}::TT *araC* P_{BAD}*fur*, Δ*pmi*, (with or without the Δ(*gmd-fcl*) mutation, see Example 18), Δ*fliC* and Δ*fljB* mutations were initially designed and constructed in strains of *S. typhimurium* to induce cross-protective immunity against *Salmonella* enterica serotypes and related enteric bacteria in chickens and other agriculturally important animals. Successes (see Examples 6, 7, 8 and 9) have led to an interest in evaluating these technologies to develop vaccines that would induce cross-protective immunity against *Salmonella enterica* serotypes and related enteric bacteria in humans. Further research concerning regulation of the *fur* gene reveals that regulation of expression is influenced by the SoxR, Crp and Fur proteins that bind to the *fur* gene promoter (Pfur) (Zheng et al., 1999, J. Bacteriol. 181:4639-4643). Therefore, in the new improved construction, the deletion of Pfur will include deletion of all promoter DNA sequences interacting with any one of these three regulatory proteins. In addition, we have identified an alternate *E. coli araC* P_{BAD} sequence (described in a patent application filed September 1, 2002 entitled "Regulated bacterial lysis for genetic vector delivery and antigen release") that gives a decreased level of transcription of genes fused to P_{BAD} when the strain is grown in medium in the absence of arabinose and will hereafter use this sequence rather than the *araC* P_{BAD} sequence used in the constructions diagramed in Figures 1 and 2 and which is present in χ8634 and its derivatives. The nucleotide sequence of this improved *araC* P_{BAD} sequence is presented in Figure 20. Figure 21 presents the nucleotide sequence of P_{fur} and the *fur* gene as found in *S. paratyphi* A and identifies the sequences within P_{fur} recognized by SoxR, Crp and Fur and the DNA sequence from *fur* -15 to *fur* -253 that will be deleted in the construction replacing P_{fur} with the improved *araC* P_{BAD} sequence given in Figure 20. This P_{fur}*fur* sequence is almost identical to that found in *S. typhimurium* and *S. typhi* such that the constructs made using *S. paratyphi* A DNA can be transferred to any of numerous *S. enterica* serotype strains. The construction of the suicide vector for introduction of the new ΔP_{fur}-33::TT *araC* P_{BAD}*fur* deletion-insertion mutation is presented in Figure 22. Since the *araC* gene is transcribed in a

direction that might generate an antisense mRNA for the adjacent *fldA* gene (see Figure 21), it is necessary to make a construction to preclude this possibility. This is because an antisense mRNA for the *fldA* gene would likely interfere with expression of the *fldA* gene and this might have unpredictable adverse effects on the vaccine strain. For this reason we insert the transcription terminator sequence *ipIII* from the bacteriophage T4 genome. The use of this and other transcription terminators for such a purpose is the subject of a patent application (US serial number 09/689,123) filed October 12, 2000. Figure 23 diagrams the chromosomal region with the ΔPfur-33::TT *araC* P_{BAD}*fur* deletion-insertion mutation with flanking DNA sequences and Figure 24 gives the entire nucleotide and encoded amino acid sequences for the multiple fusion product in the bacterial chromosome. This deletion-insertion mutation diagramed in Figure 23 can be moved to diverse strains of *S. enterica* including *S. typhimurium*, *S. paratyphi* A, and *S. typhi* using the transductional method with integrated suicide vector (see Figure 22) described by Kang et al. (2002, J. Bacteriol. 184:307-312). Bacterial strains with this mutation, when grown in medium in the presence of arabinose, will synthesize Fur protein which in turn will repress genes for the synthesis of all proteins that *Salmonella* uses to scavenge and efficiently take up iron. These strains can be fully evaluated as described in Example 4 and the synthesis of IROMPs dependent on presence or absence of arabinose in the growth medium analyzed as depicted in Figure 6. Based on the previously presented background information and results given in earlier Examples, such strains will not be subjected to iron toxicity in the intestinal tract and following oral immunization will efficiently colonize the GALT and gain access to internal lymphoid tissues in the orally immunized animal or human host. During this time, synthesis of Fur protein will cease due to the absence of arabinose in vivo and the amount of Fur protein will decrease by half at each cell division such that the vaccine strain will commence to constitutively over-express all proteins involved in iron acquisition with many of such proteins eliciting immune responses that will render animals immune to infection due to the ability of these antibodies to prevent infecting bacteria from acquiring iron, which is essential for their viability and pathogenicity.

Example 17. Improved immunogenicity with retained attenuation using regulated delayed display of attenuation.

As described in Example 6 and presented in Table 5, χ8634 with the ΔPfur-223::TT *araC* P_{BAD}*fur* insertion-deletion mutation was totally avirulent and highly immunogenic in mice. In contrast, this result is not observed in a strain with a simple Δ*fur* mutation since such Δ*fur* strains are subjected to iron toxicity in the intestinal tract leading to inefficient colonization of the GALT and internal lymphoid tissues with the result that only a low level of protective immunity to subsequent *Salmonella* challenge is induced. It is sometimes observed that some attenuating mutations render *Salmonella* totally avirulent but do not induce high-level protective immunity. In other words, the attenuating mutation does not constitute a mutation engendering high-level immunogenicity, an essential attribute of a mutation to be included in a vaccine strain that will be efficacious in inducing protective immunity.

Salmonella strains with mutations in the *rpoS* gene are highly attenuated since the RpoS gene product regulates many genes necessary for *Salmonella* to survive in stationary phase, during starvation and in response to many stresses encountered in infected animal tissues (Fang et al., 1992, Proc. Natl. Acad. Sci. USA 89:11978-11982; Wilmes-Riesenberg et al., 1997, Infect. Immun. 65:203-210). It has been demonstrated by Nickerson and Curtiss (1997, Infect. Immun. 65:1814-1823) and Coynault et al. (1996, Mol. Micribiol. 22:149-160) that *S. typhimurium* strains with mutations in the *rpoS* gene are defective in invading M cells of the follical associated epithelium (FAE) and in colonization of the GALT. Because of these properties, *Salmonella* vaccine strains with *rpoS* mutations, although attenuated, are not very immunogenic and therefore are not very efficacious in inducing protective immunity either against *Salmonella* or against protective antigens specified by cloned genes present in recombinant attenuated *Salmonella* vaccines. These negative attributes associated with the presence of *rpoS* mutations in vaccine strains are detailed in U.S. 6,024,961 and U.S. 6,383,496 that also describe means to identify, select and/or construct vaccine strains that display wild-type $RpoS^+$ phenotypes. Since the presence of a *rpoS* mutation in a vaccine strain reduces initial colonization of the GALT, there is also a reduced colonization of internal lymphoid tissues such as the mesenteric lymph node, liver and spleen that serve as major effectors sites for inducing immune responses (see Nickerson and Curtiss, 1997, Infect. Immun. 65:1814-1823). Replacement of the promoter for

the *rpoS* gene with the improved tightly regulated *araC* P_{BAD} activator-promoter sequence (Figure 20) for fusion to a promoter-less *rpoS* gene would enable synthesis of the *rpoS* gene product when the vaccine strain is grown in the presence of arabinose as would be the case for growth of the vaccine strain prior to oral immunization of an immunized individual. Such a vaccine strain would therefore contain the RpoS regulatory protein and be able to express all RpoS-regulated genes necessary for efficient invasion of M cells and colonization of the GALT. Since arabinose is not present in animal tissues, further synthesis of the *rpoS* gene product would cease and gradually RpoS would be reduced in concentration either due to cell division of the vaccine strain and/or proteolytic breakdown of the RpoS protein. In this manner, the attenuation associated with a non functioning or non expressing *rpoS* gene would be delayed until the vaccine strain had efficiently colonized internal lymphoid tissues after which the vaccine strain would become defective in responding to starvation conditions and importantly to stresses encountered in vivo. In addition, as described below in Example 19, the *rpoS* gene product is necessary for the expression of genes for synthesis of thin aggregative fimbriae, encoded by the *afg* genes, and cellulose, encoded by the *bcs* genes, that collectively constitute an extracellular matrix that is necessary for *Salmonella* to synthesize biofilms and survive in various environments into which a vaccine strain might be excreted. The fact that vaccine strains with the inactive *rpoS* gene would not survive well in stationary phase and during starvation would enhance the benefit of using a regulated delayed non expression of the *rpoS* gene to provide a biological containment attribute that would diminish vaccine survival in nature and thus decrease the likelihood for non intentional immunization of individuals either not intended or not electing to be immunized. Figure 25 provides DNA sequence information for the wild-type *S. typhimurium* 14028 and *S. typhi* CT18 *rpoS* genes (that have identical amino acid sequences) with their promoters and flanking sequences and indicates the nucleotide sequences encompassing the promoter of the *rpoS* gene (P_{rpoS}) (-12 to -48 from the ATG start of the *rpoS* gene) that will be deleted. Figure 20 provides the DNA sequence information for the improved, tightly regulated *araC* P_{BAD} sequence to be used to replace P_{rpoS}. Figure 26 diagrams the construction of the suicide vector for introduction of the ΔPrpoS-183::TT *araC* P_{BAD} *rpoS* insertion-deletion mutation into the chromosome of *Salmonella* vaccine strains. It should be noted that the T4 *ipIII* transcription terminator (TT) sequence is used after the C-terminus of the

outwardly expressing *araC* gene so that potential transcription into adjacent genes does not result in unpredictable consequences for the vaccine strain such as its further attenuation. The uses of TT sequences for this purpose and as a means of attenuation of vaccine strains are fully described in a patent application filed October 12, 2000 entitled "Microbes having an attenuating mutation comprising a transcription terminator" (US serial number 09/689,123). The deletion-insertion mutation in the chromosome is diagramed in Figure 27 and this mutation can be moved into other vaccine strains using the transductional method described by Kang et al. (2002, J. Bacteriol. 184:307-312).

Salmonella strains with Δ*phoP* and/or Δ*phoPQ* mutations are highly attenuated and induce high-level protective immunity as reported by Galan and Curtiss (1989, Microbial Pathogen. 6:433-443) and as detailed in U.S. 5,424,065 and EUR 0,465,560B1. Nevertheless, it was originally observed (Galan and Curtiss, 1989, Microbial Pathogen. 6:433-443) that although such attenuated vaccines colonized the GALT reasonably well, they did so less efficiently than did *Salmonella* strains attenuated with Δ*cya* and Δ*crp* mutations (Curtiss and Kelly, 1987, Infect. Immun. 55:3035-3043; U.S. 5,389,368). Furthermore, colonization levels by the *phoQ12* (originally designated *phoP12*) mutant in the spleen were much lower than observed for vaccine strains attenuated by the presence of other mutations (Galan and Curtiss, 1989, Microbial Pathogen. 6:433-443). Subsequently, it has been learned that bile present in the intestinal tract of animal hosts can inhibit invasion of *Salmonella* into the intestinal mucosa and into the GALT (Van Velkinburgh et al., 1999, Infect Immun. 67:1614-1622) and, furthermore, that *phoPQ* mutants are more sensitive to bile than their wild-type parents (Prouty and Gunn, 2000, Infect. Immun. 68:6763-6769). In addition, it is now known that the PhoP regulated genes *prgHIJK* specify proteins that constitute and are essential for the assembly and function, in part, of the Type III secretion apparatus (Kimbrough and Miller, 2000, Proc. Natl. Acad. Sci. USA 97:11008-11013) that is critical to the ability of *Salmonella* to successfully invade cells in the intestinal mucosa and the GALT (Kubori et al., 1998, Science 280:602-605). It is noteworthy, that the *prgHIJK* genes are within the 40 kb *inv* gene cluster originally identified by us as of critical importance for the ability of *Salmonella* to invade cells in the intestinal mucosa and the GALT (Galan and Curtiss, 1989, Proc. Natl. Acad. Sci. USA 86:6383-6387). These *inv* genes are

equally important for the ability of *Salmonella* to invade any mucosal cell surface, including the upper respiratory tract after intranasal immunization. It therefore follows, that the deletion of the promoter for the *phoPQ* operon and its replacement with the *araC* P_{BAD} activator-promoter sequence would provide a means to enhance colonization of lymphoid tissues. This is because growth of the vaccine strain in medium with arabinose prior to oral immunization of an individual would maximize the ability of the vaccine strain to survive the bile encountered in the intestinal tract and to invade into and colonize the GALT. Such more efficient invasion and colonization of the GALT would also enhance the ability of the vaccine strain to colonize internal lymphoid tissues such as the mesenteric lymph nodes, liver and spleen more efficiently prior to display of attenuation due to non expression of the *phoPQ* regulatory genes (due to the absence of arabinose in animal tissues). Figure 28 presents the nucleotide sequence of the *S. typhimurium phoPQ* operon (essentially identical to the sequences in *S. paratyphi* A and *S. typhi*) and its promoter with flanking gene sequences and indicates the nucleotides of the *phoPQ* promoter (P_{phoPQ}) deleted. Figure 20 presents the nucleotide sequence of the improved *araC* P_{BAD} activator-promoter to replace P_{phoPQ}. Figure 29 diagrams the construction of the suicide vector for the introduction of the ΔPphoPQ-107::TT *araC* P_{BAD} *phoPQ* insertion-deletion mutation into the chromosome of vaccine strains. Figure 30 diagrams the *Salmonella* chromosome with this insertion-deletion mutation. This insertion-deletion mutation can be introduced into strains of *S. typhimurium, S. paratyphi* A and *S. typhi* to be used as attenuated vaccine strains using the method of Kang et al. (2002, J. Bacteriol. 184:307-312).

Live attenuated bacterial vaccines with deletion-insertion mutations such as ΔPfur-233::TT *araC* P_{BAD} *fur*, ΔPfur-33::TT *araC* P_{BAD} *fur*, ΔPrpoS-183::TT *araC* P_{BAD} *rpoS* and ΔPphoPQ-107::TT *araC* P_{BAD} *phoPQ* will cease to express the gene fused to P_{BAD} soon after the vaccine strain is used to immunize an individual and is subject to an arabinose-free environment such that activation of the AraC protein, which requires arabinose, that is necessary to activate transcription from P_{BAD} can no longer occur. A delay in the cessation of such P_{BAD} dependent expression can be achieved by introducing the Δ*araBAD23* mutation present in χ8767 (Table 1) into the chromosome of such vaccine strains. The deletion of the *araBAD* genes for the catabolic breakdown and metabolism of arabinose causes arabinose accumulated internally by vaccine

cells during their growth in arabinose-containing media to persist and continue to be available for the activation of the AraC protein to cause transcription from P_{BAD} for an additional generation or so of growth following immunization (Guzman et al., 1995, J. Bacteriol. 177:4121-4130). This delay in onset of transcriptional shutoff can be further delayed by also introducing the Δ*araE25* mutation present in χ8477 (Table 1) that both decreases arabinose uptake into vaccine cells and also enhances its retention once internalized into the cell. Vaccine strains with both the Δ*araBAD23* and Δ*araE25* mutations are therefore grown in higher concentrations of arabinose prior to use for immunization than strains with only the Δ*araBAD23* mutation. Figure 31 diagrams the suicide vectors (listed in Table 2) for introducing the Δ*araBAD23* and Δ*araE25* mutations into the chromosomes of vaccine strains and also diagrams the mutations after being introduced into the chromosome. It should be reiterated that vaccines with any of these regulated attenuating mutations, will decrease in virulence to ultimately display total attenuation when the Fur, RpoS and/or PhoPQ proteins are diluted out as a consequence of vaccine strain cell division in vivo and/or to proteolytic breakdown of these proteins.

Strains with the ΔPrpoS-183::TT *araC* P_{BAD} *rpoS* and ΔPphoPQ-107::TT *araC* P_{BAD} *phoPQ* insertion-deletion mutations can be readily identified phenotypically. Strains with the first mutation will synthesize catalase when grown in the presence of arabinose as revealed by generation of vigorous bubbling upon addition of H_2O_2 to cultures, whereas no catalase will be synthesized by cultures grown in the absence of arabinose. This and a simple glygogen synthesis assay for revealing expression vs. non-expression of the *rpoS* gene are fully described in U.S. 6,024,961. Strains with the second mutation are readily identified by the ability to synthesize acid phosphatase encoded by the PhoP-activated gene *phoN* when strains are grown in the presence of arabinose but not when grown in the absence of arabinose. This assay is fully described in U.S. 5,424,065. PCR analyses with appropriate oligonucleotide probes can be used to rigorously validate the location and composition of the insertion-deletion mutations in the chromosomes of vaccine strains and DNA sequencing can be used to fully corroborate the presence of correct functioning DNA sequences.

The procedures described above in the Examples can be used to generate a diversity of vaccine strains that exhibit wild-type attributes during the initial immunization phase and gradually become fully attenuated the longer the vaccine strain resides in various lymphoid tissues within the immunized animal or human host. It is only necessary to delete sequences for binding of activators and repressors and the promoter that are upstream (5') to the Shine-Dalgarno (SD) ribosome binding sequence and the structural gene encoding a trait necessary for virulence of a bacterial pathogen. It is well known that mutations in the *aroA, aroC, aroD, cya, crp, cdt, ompR, htrA, hemA, purA, purB, rfa, rfb, asd, ompC,* and *ompD* genes will render bacteria such as *Salmonella* avirulent. In some of these cases, mutants with such mutations are not very immunogenic, presumably due to poor colonization of lymphoid tissues. It would therefore be logical to remove the activator and/or repressor binding sites and promoter sequence for these genes and replace this deleted sequence with an *araC* P_{BAD} sequence. In this way, the virulence gene would be expressed when the strain is growing in medium with arabinose and would gradually cease to be expressed in vivo when the vaccine strain is unable to acquire arabinose to result in attenuation (avirulence) of the vaccine strain. This means for regulated delay in display of avirulence (attenuation) has numerous benefited applications in the construction of safe, efficacious bacterial vaccines.

Example 18. Delay in cessation in LPS O-antigen side chain synthesis in a vaccine strain with the Δ*pmi*-2426 mutation by blocking the conversion of GDP-Mannose to GDP-Fucose by inclusion of the Δ(*gmd-fcl*)-26 mutation.

As shown by data presented in Tables 5, 6 and 7 (Example 6), the strain χ8754 with both the ΔPfur-223::TT *araC* P_{BAD} *fur* and Δ*pmi-2426* mutations was somewhat less immunogenic than strains with either insertion-deletion or deletion mutation alone. Strains with *pmi* mutations are unable to use mannose as an energy source but are able to take it up into the cell, phosphorylate it and convert it to GDP-Mannose, one of the substrates necessary for synthesis of the LPS O-antigen side chains present in almost all *Salmonella* serotypes. However, *Salmonella* and other enteric bacteria can synthesize the exopolysaccharide colanic acid that is often synthesized in response to stresses. Fucose makes up one-third of the mass of colanic acid and is

incorporated into this polymer using GDP-Fucose as a substrate (Grant et al., 1970, J. Bacteriol. 103:89-96). GDP-Fucose is synthesized from GDP-Mannose in two steps catalyzed by two enzymes encoded by the *gmd* and *fcl* genes (Andrianopoulos et al., 1998, J. Bacteriol. 180:998-1001). Therefore, a vaccine strain with the Δ*pmi-2426* mutation alone might use some of the mannose taken up from the medium during its growth prior to immunization of an individual to be diverted to the synthesis of colanic acid by the conversion of GDP-Mannose to GDP-Fucose rather than use all of the accumulated GDP-Mannose to synthesize LPS O-antigen side chains. This would have the impact of causing a more rapid cessation in synthesis of LPS O-antigen side chains after immunization with a more rapid onset of the attenuating features associated with the presence of the *pmi* mutation. We have constructed a suicide vector with the Δ(*gmd-fcl*)-*26* mutation that deletes both genes encoding enzymes for the conversion of GDP-Mannose to GDP-Fucose (Figure 32; Table 2) and used it to introduce the Δ(*gmd-fcl*)-*26* mutation (diagramed in Figure 33) into the wild-type *S. typhimurium* UK-1 strain χ3761 and into the Δ*pmi-2426* containing UK-1 strain χ8650 to yield χ8831 and χ8868, respectively. As evidenced by the data in Table 10, the Δ(*gmd-fcl*)-*26* mutant has the same virulence as the UK-1 wild-type parent χ3761 and the two strains with the Δ*pmi-2426* mutation are equally attenuated independent of the presence or absence of the Δ(*gmd-fcl*)-*26* mutation. (Table 10 needs to be inserted after it is first cited in text.) This result is important in demonstrating that the presence of the Δ(*gmd-fcl*)-*26* mutation that precludes conversion of GDP-Mannose to GDP-Fucose does not result in the buildup of a pool of GDP-Mannose sufficient to cause the double mutant to demonstrate some lethal infections in mice at high doses. Table 10 also presents data to show that χ8650 and χ8868 have essentially equal immunogenicity when the vaccine strains are grown in Luria broth (which contains 0.1% glucose) with 0.5 % mannose prior to oral immunization of mice with decreasing doses of vaccine and challenged with high 10^9 CFU doses of the wild-type χ3761 thirty days later. Based on these results, the Δ(*gmd-fcl*)-*26* mutation will be included in all vaccine strains with the Δ*pmi-2426* mutation that are designed to be used to induce cross-protective immunity against *S. enterica* serotypes and other related enteric bacterial pathogens.

TABLE 10. Virulence of *S. typhimurium* UK-1 strains with Δ(*gmd-fcl*)-*26*, Δ*pmi-2426*, and Δ(*gmd-fcl*)-*26* Δ*pmi-2426* mutations in 8-week-old female BALB/c mice following oral

inoculation and protectiv immunity by strains with *Δpmi-2426* with and without th *Δ(gmd-fcl)-26* mutation[a]

Strain	Inoculating dose	Survivors/ total	Challenge dose	Survivors/total after challenge
χ3761 (wild type)	1.2×10^7	0/4		
χ8831 *Δ(gmd-fcl)-26*	1.0×10^5	1/4	ND	ND
	1.0×10^4	4/4		
	1.0×10^3	4/4		
χ8650 *Δpmi-2426*	1.1×10^9	4/5	1.2×10^9	4/4
	1.1×10^8	4/5	1.2×10^9	3/4
	1.1×10^7	5/5	1.2×10^9	3/5
	1.1×10^6	5/5	1.2×10^9	2/5
χ8868 *Δpmi-2426* *Δ(gmd-fcl)-26*	1.1×10^9	5/5	1.2×10^9	4/5
	1.1×10^8	4/5	1.2×10^9	4/4
	1.1×10^7	5/5	1.2×10^9	3/5
	1.1×10^6	4/5	1.2×10^9	0/4

[a] Bacteria were grown in Luria broth (containing 0.1% glucose) supplemented with 0.5% mannose to OD_{600} of~0.8. Bacterial cells were collected by centrifugation and suspended in buffered saline with gelatin (BSG). Female BALB/c mice, 8-weeks-old, were orally inoculated with 20 µl of the bacterial suspension. Morbidity and mortality were observed for 30 days. Surviving mice were challenged 30 days after the initial inoculation with virulent wild-type UK-1 χ3761 grown in Luria broth. Morbidity and mortality observations were recorded daily for an

additional 30 days postchallenge. Both inoculating and challenge doses were measured in CFU.

Example 19. Diminishing the ability of vaccine strains designed to induce cross-protective immunity against enteric bacterial pathogens to persist in vivo and/or be shed and persist in the environment.

Live attenuated *Salmonella* vaccines used to prevent infection of broiler chickens with *Salmonella* and to diminish, if not eliminate, presence of pathogenic *Salmonella* on carcasses at slaughter should be designed to not persist in immunized animals for more than about three weeks after receiving the last immunizing dose. Broilers now go to market at about six weeks of age and receive a second booster immunization with live attenuated *Salmonella* vaccines at 10 to 14 days of age. This feature is not so important when using such vaccines to immunize larger animals including swine, calves, cattle, goats, sheep, turkeys and chickens raised as roasters or to supply meat for the "nugget" market that are slaughtered at a more advanced age than broiler chickens. On the other hand, persistence of live attenuated vaccine strains in the intestinal tract of immunized animals leads to their excretion in feces with the potential to contaminate and persist in various environmental niches. This is also undesirable since such surviving vaccines might cause immunization of individuals either not intended to be vaccinated or, in the case of human animal caretakers, not electing to be immunized. A further negative to potential persistence of vaccine strains in agricultural environments, would be to diminish need by producers/farmers to purchase new lots of vaccine to immunize every new lot of animals, and such a feature would dissuade commercial development and marketing of such live attenuated vaccines. In regard to these issues, note that the live attenuated vaccine strain χ8754 still demonstrates detectable low titers in mice (Figure 9) 42 days after immunization. To address these concerns, we have and are continuing to develop genetic strategies to provide live attenuated bacterial vaccine strains with biological containment features to lessen their ability to persist in vivo and to survive in natural environments likely encountered if shed in feces.

It is most desirable that mutations that confer desirable biological containment features not attenuate infectivity of vaccine strains and permit the same level of initial colonization of lymphoid tissues as the attenuated vaccine strain without the mutation conferring biological containment. This is invariably the case if a wild-type virulent strain endowed with the mutation conferring biological containment has an LD_{50} that is nearly identical to its wild-type parent. We have therefore used this parameter to initially select mutations that can or do provide biological containment that do not diminish infectivity and virulence.

Strains with mutations such as Δ*fliC825* and Δ*fljB217* are non-flagellate and are non-motile. These mutations have been introduced into live attenuated *Salmonella* vaccines to induce cross-protective immunity to diverse enteric bacterial pathogens since antibody responses to the FliC and FljB protein antigens are serotype specific and thus would be unimportant in inducing cross-protective immunity. Since *Salmonella* in polluted aqueous environments uses motility and chemotaxis to identify food sources and swim toward them, non-motile strains with Δ*fliC* and Δ*fljB* mutations would be less able to survive in nature due to an inability to identify and move toward food supplies. It should be noted that chemotaxis is also dependent on the presence of flagella and display of motility. A bacterial strain such as χ8602 (Table 1) has the Δ*fliC825* and Δ*fljB217* mutations. It is non-flagellate and non-motile and, importantly, has the same LD_{50} as does its wild-type parent χ3339 (Table 11).

Table 11. Virulence of *S. typhimurium* strains with deletion and deletion-insertion mutations contributing to biological containment.

Strain	Genotype	CFU/dose	Survival/total
χ3761	UK-1 wild-type	1×10^7 1×10^6 1×10^5	0/2 1/5 1/5
χ3761	wild-type	1.5×10^6 1.5×10^5 1.5×10^4 1.0×10^3	0/4 1/4 3/4 4/4
χ3761	wild-type	9×10^5 9×10^4	0/4 2/4

χ8894	Δ*adrA1418*	1.1×10^8 1.1×10^7 1.1×10^5	0/3 1/3 0/3
χ8890	Δ*bcsABZC2118*	1.5×10^8 1.5×10^7 1.5×10^5	0/3 0/3 1/3
χ8892	Δ*bcsEFG2319*	2.1×10^9 2.1×10^7 2.1×10^5	0/3 0/3 1/3
χ8844	Δ*endA2311*	8.6×10^6 8.6×10^5 8.6×10^4	0/4 2/4 2/2
χ8844	Δ*endA2311*	3.0×10^5 3.0×10^4	0/2 1/2
χ8831	Δ(*gmd-fcl*)-*26*	5.9×10^5 5.9×10^4 5.9×10^3 5.9×10^2	1/4 4/4 4/4 4/4
χ8831	Δ(*gmd-fcl*)-*26*	8.6×10^6 8.6×10^5 8.6×10^4 8.6×10^3	0/4 0/4 0/4 1/4

Table 11. (cont'd)

Strain	Genotype	CFU/dose	Survival/total
χ8882	Δ*relA1123m*	8.0×10^7 8.0×10^6 8.0×10^5 8.0×10^4 8.0×10^3	0/4 1/5 1/4 3/4 4/4
χ8857	Δ*yhiR36*::TT	2.0×10^6 2.0×10^5 2.0×10^4	 1/4 4/4
χ3339	SL1344 wild-type	1.0×10^6	0/4
χ8602 SL1344	Δ*fliC825* Δ*fljB217*	2.9×10^6 2.9×10^5 2.9×10^4	0/4 1/4 4/4

As discussed in Example 18, enteric bacteria are capable of synthesizing the exopolysaccharide colanic acid in response to stresses. The presence of colanic acid can enhance resistance to antibiotics and other anti-microbial drugs, enhance resistance to host defense mechanisms including attach by lysozyme, complement and phagocytes, and also confers enhanced resistance to death by desiccation (Lopez-Torres and Stout, 1996, Curr. Microbiol. 33:383-389)). The presence of the Δ(*gmd-fcl*)-*26* mutation in vaccine strains would not only have the benefits described in Example 18, but would also contribute to the biological containment features of the vaccine. As presented in Table 10, χ8831 with the Δ(*gmd-fcl*)-*26* mutation is as virulent as its wild-type parent χ3761.

Synthesis of the extracellular matrix composed of thin aggregative fimbriae (curli) and cellulose (Romling et al., 2001, Mol. Microbiol. 39:1452-1463) enables enteric bacteria to synthesize biofilms that enhance their ability to adhere to both biological and inanimate surfaces, that is to colonize and survive on these surfaces that are encountered in the intestinal tract and in the environment following excretion. We have thus constructed the Δ*afgBAC811* mutation to abolish synthesis of thin aggregative fimbriae and introduced it into χ3339 to produce strain χ8606 (Table 1). We have also generated the Δ*bcsABZC2118* and Δ*bcsEFG2319* mutations to abolish ability to synthesize cellulose (Solano et al., 2002. Mol. Microbiol. 43:793-808) and introduced both mutations into χ3761 to produce χ8890 and χ8892, respectively (Table 1). As described in Example 17, synthesis of the extracellular matrix can also be abolished by various other mutations in regulatory genes. We thus constructed the Δ*adrA1418* mutation that blocks the export of cellulose to the cell surface (Zogaj et al., 2001, Mol. Microbiol. 39:1452-1463; Romling et al., 2001, Mol. Microbiol. 36:10-23), even when there are no mutations in *bcs* genes, to generate strain χ8894. Strains with mutations in the *mlrA* gene (Brown et al., 2001, Mol. Microbiol. 41:349-363) are unable to synthesize either thin aggregative fimbriae or to export cellulose to the cell surface (since MlrA is necessary to express the *adrA* gene). A χ3339 derivative with a mutation in the *mlrA* gene, χ8702, is listed in Table 1. Data presented in Table 11 reveals that *S. typhimurium* strains with the Δ*afgBAC811* (χ8606), Δ*bcs*ABZC2118 (χ8890),

Δ*bcsEFG2319* (χ8892), Δ*adrA1418* (χ8894) and *mlrA34* (χ8702) mutations retain the virulence with similar LD_{50} values as exhibited by their wild-type virulent parents. Thus these mutations preventing complete synthesis of the extracellular matrix are non attenuating.

Finkel and Kolter (2001, J Bacteriol. 183:6288-93) demonstrated that *E. coli* could use exogenous DNA as a nutrient to survive during prolonged stationary phase growth and then found that a mutant strain with a mutation in the *yhiR* gene was less able to use DNA as a nutrient and thus survived very poorly during prolonged stationary phase growth in comparison to the wild-type parent. We have therefore generated the Δ*yhiR36*::TT mutation and introduced it into χ3761 to produce χ8857 (Table 1). In initial experiments during mixed cultivation, χ8857 only constituted 18 % of the surviving bacterial population after four days in comparison to 82 % for the wild-type strain. Since enteric bacteria have endonuclease I in their periplasmic space and could use this enzyme to initially degrade either linear or circular DNAs that might serve as nutrients, we generated the Δ*endA2311* mutation and introduced it into χ3761 to yield χ8844 (Table 1) and into χ8857 to yield χ8865 with both Δ*yhiR36* and *endA2311* mutations (Table 1). χ8857, χ8854 and χ8865 all exhibit virulence similar to the wild-type parents (Table 11).

Enteric bacteria when subjected to nutrient starvation invoke a stringent regulatory response and shut down protein synthesis. This causes a cessation of any attempt at growth or cell division and thus invokes a "Rip van Winkle" type of survival response. To preclude this survival capability, we have generated the Δ*relA1123* mutation, since *relA* mutations uncouple the ability of bacteria to respond to starvation signals. Thus nutrient limitation results in continued attempts at macromolecular synthesis and growth and this unbalanced growth enhances the likelihood for cell death. χ8882 with the Δ*relA1123* mutation (Table 1) may exhibit a very low level of attenuation compared to its wild-type parent (Table 12).

Figure 34 diagrams all the suicide vectors (listed in Table 2) for introducing each of the above-described mutations into the chromosomes of *Salmonella* vaccine strains to confer biological containment properties to the vaccine strains. Figure 35 diagrams all the mutations

after insertion into the chromosome. The transductional method of Kang et al. (2002, J. Bacteriol. 184:307-312) can be used to easily move these markerless deletion mutations to other bacterial vaccine strains being constructed. Some or all of these mutations can be included in any one strain to provide biological containment. This is facilitated by the fact that there are no antibiotic resistance genes or other selective markers needed to select for inheritance of the markerless deletion mutation being introduced into any vaccine strain. This is also desirable since expression of antibiotic resistance by live attenuated bacterial vaccines would be unsafe if not unethical and is usually not permitted by regulatory agencies charged with evaluation and licensing of live attenuated bacterial vaccines.

An additional independent means to achieve essentially total biological containment of live attenuated bacterial vaccines is the subject on an independent patent application filed on 9-01-02 entitled "Regulated bacterial lysis for genetic vector delivery and antigen release." The technologies described in that application can be used to confer a most complete type of biological containment on vaccine strains since vaccine cells ultimately all die due to their lysis either in vivo or shortly after their excretion.

Example 20. Generation of *sopB* mutations so that live attenuated *S. typhimurium* vaccines used to orally immunize humans to induce cross-protective immunity against enteric bacterial pathogens will not induce gastroenteritis (diarrhea) as a consequence of immunization.

We anticipate evaluating a genetically modified live attenuated *S. typhimurium* vaccine with the ΔPfur-33::TT *araC* P_{BAD} *fur*, Δ*pmi-2426*, Δ(*gmd-fcl*)*-26*, Δ*fliC825*, Δ*fliC2426* or Δ*fliC*-Var, Δ*fljB217*, and a selected optimal array of deletion mutations to provide biological containment properties to the vaccine for immunization of humans to evaluate induction of cross-protective immunity to diverse enteric bacterial pathogens. *S. enterica* strains, including *S. typhimurium*, are frequently the cause of gastroenteritis in humans with associated diarrhea and other unpleasantries. We surmise that the live attenuated *S. typhimurium* vaccine strain with the above listed mutations would be capable of inducing such disease, at least in some vaccinees,

since it is still invasive and colonizes all lymphoid tissues, at least in mice. Various studies have implicated the effector proteins SopA, SopB, SopD and SopE2 as responsible for the induction of fluid secretion in animals susceptible to *S. enterica* induced gastroenteritis (Paesold et al., 2001, Annual Meeting of the Federation of American Society for Experimental Biology on Experimental Biology, P. A825; Zhang et al., 2002, Infect. Immun. 70:3843-3855). These proteins, encoded by genes in various regions of the chromosome, are all delivered to the cytoplasm of host cells in the infected individual by the Type III secretion system encoded in *Salmonella* Pathogenicity Island 1 (SPI-1) that contains the genetic information essential for *Salmonella* invasion into mucosal tissues (Galan and Zhou, 2000, Proc. Natl. Acad. Sci. USA 97:8754-8761; Galan, 2001, Annu. Rev. Cell Dev. Biol. 17:53-86). Various mutations will block the ability of *S. typhimurium* and *S. dublin* to cause fluid secretion resulting in diarrhea, but many of these mutations, such as in the *sipB* gene, yield strains that are non-invasive and unable to induce apoptosis and are therefore likely to be non-immunogenic. We will therefore construct a defined deletion mutation of the *sopB* gene that encodes an inositol phosphate phosphatase since the absence of this gene results in the most substantial reduction in fluid secretion compared to a *sipB* mutant (Paesold et al., 2001, Annual Meeting of the Federation of American society for Experimental Biology on Experimental Biology, P. A825; Zhang et al., 2002, Infect. Immun. 70:3843-3855) without reducing invasion ability. Figure 36 provides the nucleotide and amino acid sequences of the *S. typhimurium sopB* gene and specifies the extend of the deletion to be present in the suicide vector diagramed in Figure 37 for introducing the Δ*sopB1925* mutation into the chromosome of *Salmonella* vaccine strains. The oligonucleotide primers to generate the deletion and to construct the suicide vector are given in Figure 37. Figure 38 provides a diagram of this Δ*sopB1925* mutation in the *S. typhimurium* chromosome along with flanking genes. The Δ*sopB1925* mutation will initially be introduced into the wild-type *S. typhimurium* UK-1 χ3761 strain to fully evaluate its virulence in mice, invasiveness into cells in culture and inability to induce fluid secretion using the ligated ilial loop assay in rabbits (that are highly susceptible to *S. enterica* induced diarrhea). We anticipate that virulence and invasiveness will be closely similar to these attributes displayed by the wild-type χ3761 parent whereas fluid secretion in the rabbit will be minimal compared to the wild-type parent. The Δ*sopB1925* mutation will then be introduced into a live attenuated *S. typhimurium* vaccine strain that is highly immunogenic to

determine whether the vaccine strain with the Δ*sopB* mutation is as immunogenic as its parent. If it is, we will introduce the Δ*sopB1925* mutation into the vaccine strains derived from *S. typhimurium,* and also derived from *S. paratyphi* A and *S. typhi* (see Example 21 below), to induce cross-protective immunity to pathogenic enteric bacterial pathogens. If the presence of the Δ*sopB1925* mutation introduces undesired attributes to the vaccine strain, we will proceed to evaluate use of Δ*sopE2*, Δ*sopD* and Δ*sopA* mutations (in that order) to arrive at the optimal balance between invasivness and colonization of lymphoid tissues to engender high immunogenicity and decreased ability to cause gastroenteritis. The goal is a safe, efficacious vaccine that will be "user friendly".

Example 21. Construction of live attenuated *S. paratyphi* A and *S. typhi* vaccines for optimal induction of cross-protective immunity against enteric bacterial pathogens.

Since there is little information that would validate the concept that a live attenuated *S. typhimurium* vaccine to induce cross-protective immunity to diverse enteric bacterial pathogens would be efficacious in humans, it is appropriate to also construct and evaluate (in human volunteers) human host-adapted *S. paratyphi* A and *S. typhi* vaccines for this purpose. Such a vaccine derived from *S. paratyphi* A would be particularly beneficial since there is currently no live attenuated vaccine to protect against *S. paratyphi* A infection that results in enteric fever with considerable global morbidity and mortality. We will use a well-characterized *S. paratyphi* A strain, χ8387, that we derived from ATCC 9281. As the *S. typhi* parents we will use both our RpoS$^+$ derivative of *S. typhi* Ty2, χ8438 (see U.S. 6,383,496), and the RpoS$^+$ *S. typhi* ISP1820 strain χ3744. These parent strains are listed in Table 1. Using suicide vectors listed in Table 2, individual strains with each defined deletion or insertion-deletion mutation in its chromosome as listed in Table 1 and the transductional method for introducing markerless mutations into the chromosome of bacterial strains (Kang et al., 2002, J. Bacteriol. 184:307-312), we will construct derivatives of χ8387, χ8438 and χ3744 that possess the ΔPfur-33::TT *araC* P_{BAD} *fur*, Δ*pmi-2426*, Δ(*gmd-fcl*)*-26*, Δ*fliC825* or Δ*fliC*-Var, Δ*fljB217*, and a selected optimal array of deletion

mutations to provide biological containment. We will also introduce the Δ*sopB1925* (or other Δ*sop* mutation, if necessary) into each strain. This is due to the widespread observation that some 10 to 15 percent of vaccinees receiving a candidate attenuated *S. typhi* vaccine have diarrhea. Thus introducing a *sopB* mutation would eliminate this problem. Constructed strains will be fully characterized phenotypically and genotypically by all the relevant procedures described in the preceding Examples. Since there is no animal model to evaluate *S. paratyphi* A and *S. typhi* candidate vaccines, evaluation for safety and efficacy will require evaluation in human volunteers. Animal data correlated with induced antibody titers monitored by the modified ELISA method described in Example 15 will, however, be instructive in evaluating antibodies induced in humans in relation to their likely ability to induce cross-protective immunity to diverse enteric bacterial pathogens.

Example 22. Use of live attenuated *Salmonella* vaccines inducing cross-protective immunity to enteric bacterial pathogens or displaying regulated delayed display of attenuation as recombinant attenuated vaccine antigen delivery vectors to induce immunity to more distantly related enteric pathogens using functional balanced-lethal host-vector constructions.

Live attenuated *Salmonella* vaccines are very useful as antigen delivery vectors to induce protective immunity to pathogens whose genes for protective antigens are contained within and expressed by the live recombinant attenuated vaccine. These technologies are described in U.S. 5,888,799. The stable maintenance and high-level expression of cloned genes on plasmid vectors by these live recombinant attenuated *Salmonella* vaccines in vivo following immunization of an animal or human host is achieved by using a balanced-lethal host-vector system as fully described in U.S. 5,672,345 and in a pending application filed October 11, 2000 entitled "Functional balanced-lethal host-vector system" (US serial number 09/868,499). In these vaccine constructs, the chromosome of the vaccine strain possesses a mutation such as Δ*asdA16* that imposes an obligate requirement for diaminopimelic acid (DAP), an essential constituent of the rigid layer of the bacterial cell wall, an amino acid that is only synthesized by bacteria and that is unavailable in animal tissues. In the absence of DAP, a strain with an *asd* mutation (or other

mutation imposing a requirement for DAP) will outgrow its wall due to DAP-less death, which occurs by cell lysis. This system is operable as a vaccine if the plasmid vector encoding a protective protein antigen from some pathogen possess a wild-type copy of the *asd* gene (or a wild-type homolog to the mutated chromosomal gene imposing the requirement for DAP) such that a complementation heterozygote is established. In this case, so long as the plasmid vector with the wild-type complementing gene is maintained in the mutant attenuated bacterial vaccine, the recombinant vaccine will survive in vivo and continue producing the protective antigen as a factory to continuously stimulate the immunized host to elicit immune responses that will later protect the immunized host against infection by the pathogen whose protective antigen was synthesized and delivered to the host by the live recombinant attenuated vaccine. Figure 39 diagrams two suicide vectors (Table 2) for introducing the Δ*asdA16* mutation into the *S. typhimurium* chromosome and the Δ*asdA25* mutation into the *S. paratyphi* A and *S. typhi* chromosomes. The necessity for two suicide vectors is due to the existence of a 24 base pair difference and an additional 30 base pair insertion adjacent to the *asd* gene in the human host-adapted *S. paratyphi* A and *S. typhi* strains that are not present adjacent to the *asd* gene in *S. typhimurium.* Figure 40 diagrams the mutations and flanking sequences within the chromosomes of the three *Salmonella* serotypes. The transductional procedure of Kang et al. (2002, J. Bacteriol. 184:307-312) can be used to move the Δ*asdA16* and Δ*asdA25* mutations to other strains such as those with the insertion-deletion mutations ΔPfur-33::TT *araC* P_{BAD} *fur*, ΔPrpoS-183::TT *araC* P_{BAD} *rpoS* and ΔPphoPQ-107::TT *araC* P_{BAD} *phoPQ* causing regulated delayed expression of attenuation. These strains would be used in conjunction with Asd^+ plasmid vectors (Figure 41) modified to specify synthesis of protective antigens from other pathogens. Figure 42 gives the nucleotide sequence of the P_{trc} promoter and the multiple cloning sites useful for such constructions with these Asd^+ vectors. Although the technology is applicable to expressing protective antigens from any pathogen, special attenuation would be focused on enhancing induction of immunity to enteric bacterial pathogens that are unrelated or not closely related to *Salmonella*. These might therefore include expression of protective antigens from enteric bacterial pathogens such as *Campylobacter jejuni*, *Listeria monocytogenes*, *Shigella* species, *E. coli* strains, *Enterococcus* species, *Clostridium* species, etc. In these cases, the host strain would also contain mutations to enhance induction of cross-protective immunity such as the ΔPfur-

33::TT *araC* P_{BAD}*fur*, Δ*pmi-2426*, Δ(*gmd-fcl*)-26, Δ*fliC825*, Δ*fliC2426* or Δ*fliC*-Var, Δ*fljB217* and selected mutations to enhance biological containment properties.

Example 23. Regulated delayed over-expression of the Type I fimbrial adhesin protein FimH to enhance induction of cross-protective immunity against enteric bacterial pathogens.

The FimH adhesive protein on type 1 fimbriae is antigenically and structurally conserved (Abraham et al., 1988, Nature 336:682-684) and the amino acid sequence of the protein in all *S. enterica* serotypes for which sequence information exists are 98 to 99 percent identical (based on GenBank analysis). Therefore, the induction of a strong immune response, especially a mucosal immune response, will likely contribute significantly to the induction of cross-protective immunity. We (Lockman and Curtiss, 1990, Infect. Immun. 58:137-143; Lockman and Curtiss, 1992, Infect. Immun. 60:491-496; Lockman and Curtiss, 1992, Mol. Microbiol. 6:933-945) had previously found that bacterial cells expressing type 1 fimbriae were unaltered in virulence and colonizing ability but were more rapidly cleared from blood than mutants unable to synthesize type 1 fimbriae. Such cells are more susceptible to phagocytosis (Ofek and Sharon, 1988, Infect. Immun. 56:539-547) and may also be more rapidly cleared from lymphoid tissues. These attributes might be intensified with a vaccine strain genetically altered to over express either type 1 fimbriae or just the FimH adhesive protein, which in either case could lead to hyper attenuation of the vaccine strain to reduce its immunogenicity. We have therefore devised a means to construct a live recombinant attenuated *Salmonella* vaccine that will give a delayed over expression of the *S. typhimurium* FimH protein after the vaccine strain has colonized lymphoid tissues. We have Asd^+ vectors pYA3337 with the low copy number pSC101 *ori*, pYA3332 with the moderately low p15A *ori*, pYA3342 with the moderate to high pBR *ori* and pYA3341 with the high copy number pUC *ori*. All of these Asd^+ vectors that are diagramed in Figure 41 have the P_{trc} promoter to drive expression of genes cloned into the multiple cloning site whose sequence (that is the same in all four vectors) is presented in Figure 42. Transcription from P_{trc} promoter is repressed (prevented) if the LacI repressor protein is present in the cytoplasm of the bacterial cell. To achieve this, we have constructed as diagramed in Figure 43 the insertion-

deletion mutation Δ*ilvG3*::TT *araC* P_{BAD} *lacI* TT and a suicide vector for its introduction into the chromosome of vaccine strains. When strains with this insertion-deletion mutation are grown with arabinose in the medium, LacI protein is synthesized. After immunization, LacI protein decreases in concentration as a consequence of cell division and the degree of repression of a P_{trc} promoter would gradually decrease with an eventual high-level constituitive expression of any gene sequence controlled by P_{trc}. Further delay in de-repression of genes controlled by P_{trc} on Asd^+ vectors can be achieved, as describe in Example 17, by introducing into the vaccine strain the Δ*araBAD23* and Δ*araE25* deletion mutations using the suicide vectors diagramed in Figure 31. Figure 44 provides the nucleotide and amino acid sequences of the *S. typhimurium fimH* gene and FimH protein. The strategy, using PCR and the listed oligonucleotide probes to clone either the entire *fimH* gene or a sequence specifying its first 100 amino acids into any of the Asd^+ vectors diagramed in Figure 41 using the multiple cloning site diagramed in Figure 42, is diagramed in Figure 45. It is known that the first 100 amino acids of the FimH protein specify the adhesive properties of type 1 fimbriae (Thankavel et al., 1997, J. Clin. Invest. 100:1123-1126) and that immune responses to this 100 amino acid sequence block adherence of type 1 expressing bacteria to host cells possessing the receptor for type 1 fimbriae (). Construction of simple attenuated vaccine strains with either of the two *fimH* inserts into anyone of the four Asd^+ vectors introduced into strains with the Δ*ilvG3*::TT *araC* P_{BAD} *lacI* TT and with and without the Δ*araBAD23* and Δ*araE25* mutations will lead to comparative studies on the stability, colonizing ability and immunogenicity of each construct. A construction with the best attributes and inducing high mucosal and systemic antibody titers against FimH that block type 1 fimbriae-mediated adherence will be the basis for further modification and enhancement of a vaccine with other insertion-deletion and deletion mutations demonstrated to maximize induction of cross-protective immunity against enteric bacterial pathogens.